Targeting Aberrant Sialylation in Cancer Cells Using a Fluorinated Sialic Acid Analog Impairs Adhesion, Migration, and In Vivo Tumor Growth

Christian Büll1, Thomas J. Boltje2, Melissa Wassink1, Annemarie M.A. de Graaf1, Floris L. van Delft2, Martijn H. den Brok1, and Gosse J. Adema1

Abstract
Cancer cells decorate their surface with a dense layer of sialylated glycans by upregulating the expression of sialyltransferases and other glycoenzymes. Although sialic acids play a vital role in many biologic processes, hypersialylation in particular has been shown to contribute to cancer cell progression and metastasis. Accordingly, selective strategies to interfere with sialic acid synthesis might offer a powerful approach in cancer therapy. In the present study, we assessed the potential of a recently developed fluorinated sialic acid analogue (P-3Fax-Neu5Ac) to block the synthesis of sialylglycans in murine melanoma cells and the consequences on cell adhesion, migration, and in vivo growth. The results showed that P-3Fax-Neu5Ac readily caused depletion of α2,3- and α2,6-linked sialic acids in B16F10 cells for several days. Long-term inhibition of sialylation for 28 days was feasible without affecting cell viability or proliferation. Moreover, P-3Fax-Neu5Ac proved to be a highly potent inhibitor of sialylation even at high concentrations of competing sialyltransferase substrates. P-3Fax-Neu5Ac-treated cancer cells exhibited impaired binding to poly-L-lysine, type I collagen, and fibronectin and diminished migratory capacity. Finally, blocking sialylation of B16F10 tumor cells with this novel sialic acid analogue reduced their growth in vivo. These results indicate that P-3Fax-Neu5Ac is a powerful glycomimetic capable of inhibiting aberrant sialylation that can potentially be used for anticancer therapy. Mol Cancer Ther; 12(10); 1935–46. ©2013 AACR.

Introduction
Overexpression of sialyltransferases and other glycoenzymes during malignant transformation and progression results in aberrant sialylation of cancer cells (1–3). The high expression of sialic acids can protect cancer cells from apoptosis, promote metastasis, and has been suggested to confer resistance to therapy (4–8). Despite increasing amounts of evidence showing the involvement of sialyltransferases and aberrant sialylation in cancer progression, therapeutic strategies to reduce aberrant sialylation lag behind. So far, sialidases or compounds that block enzymes in sialic acid synthesis have been tested with limited success. Enzymatic removal of sialic acids with bacterial sialidases only leads to short-term depletion of cell surface sialic acids. Plant-derived or synthetic inhibitors of the sialic acid machinery are rare and show low efficacy (9). The rational design of sialic acid analogues that interfere with the cellular sialic acid synthesis machinery might form a more promising approach (10–12). Here sialyltransferases constitute an attractive target as they have been shown to underlie aberrant cancer sialylation. Sialyltransferases are expressed in the Golgi system where they incorporate sialic acid residues into assembling glycan trees of cell surface glycoproteins and lipids (13). Up to date, more than 20 different human and murine sialyltransferases have been identified, each attaching sialic acids via distinct glycosidic linkages (e.g., α2,3 or α2,6) to various monosaccharide residues like galactose (13, 14). Hence, upregulation of sialyltransferases results in the expression of highly sialylated structures including sialylglycoproteins, sialogangliosides, or sialyl Lewis a or x (SLea/x) antigens. These sialylated structures are a hallmark of many cancer cells and have been shown to contribute to numerous processes in cancer growth (15–19). For example, overexpression of ST6Gal-I and increased α2,6-sialylation of the Fas receptor mediates resistance to Fas-induced apoptosis (4). Hypersialylation (α2,6) of αvβ3 integrin enhances binding to collagen type I and favors cancer cell migration (20). Perea-Garay and colleagues reported that ST3Gal-III–overexpressing pancreatic adenocarcinoma cells formed metastatic lesions and drastically decreased survival in mice (21).
Recently, Rillahan and colleagues have described the synthesis of a global metabolic inhibitor of STs, P-3Fax-Neu5Ac (22). P-3Fax-Neu5Ac has been shown to efficiently inhibit sialylation following uptake by human leukemia cells. Structurally, P-3Fax-Neu5Ac resembles N-acetylneuraminic acid (Neu5Ac), the most common sialic acid derivate and the endogenous substrate for Golgi-resident sialyltransferases. During chemical synthesis, Neu5Ac is converted to a methyl ester and peracylated (P-Neu5Ac) to enhance cellular uptake and subsequently an axial fluorine atom is incorporated at the C-3 carbon via the glycal to yield P-3Fax-Neu5Ac (Fig. 1A). P-3Fax-Neu5Ac presumably enters the intracellular sialic acid synthesis pathway via a salvage pathway and is converted to CMP-3Fax-Neu5Ac (23). Only CMP-activated sialic acids can be recognized by the CMP-sialic acid transporter and undergo active transport into the Golgi lumen where they serve as substrate for sialyltransferases (24, 25). Rillahan and colleagues proposed that P-3Fax-Neu5Ac blocks sialylation globally via 2 mechanisms. Initially, CMP-activated P-3Fax-Neu5Ac inhibits sialyltransferases directly and thereby prevents incorporation of natural sialic acids into assembling glycans. As a consequence, CMP sialic acids accumulate in the cell and disable UDP-N-acetylglucosamine 2-epimerase/ N-acetylmannosamine kinase (GNE/MNK), a key enzyme upstream in the de novo synthesis pathway of sialic acids (25, 26). In a 2-step reaction, GNE/MNK catalyzes intracellular sialic acid precursors (UDP-N-Acetylglucosamine) to N-Acetylmannosamine (ManNAc) and ManNAc-6-phosphate (ManNAc-6P) which is finally converted into CMP-Neu5Ac (27). This reaction is tightly regulated as CMP-Neu5Ac can inhibit GNE/MNK activity to prevent excessive sialylation.

Together, blocking sialyltransferases directly as well as blocking the synthesis of sialic acids indirectly makes P-3Fax-Neu5Ac a global inhibitor of sialylation that could be of high value for glycobiological research and moreover could be used to therapeutically restore aberrant sialylation of cancer cells. Therefore, we have assessed for the first time the effects and kinetics of P-3Fax-Neu5Ac on aberrant tumor cell sialylation as well its ability to modulate adhesion, migration, and in vivo growth of murine cancer cells.

Materials and Methods

Mice
Female C57BL/6J mice, 6 to 8 weeks old at the beginning of the experiment, were obtained from Harlan Laboratories. Mice were housed in the Central Animal Laboratory, Radboud University, Nijmegen, The Netherlands, under specific pathogen-free conditions. All experiments were authorized by the local animal ethics committee, Nijmegen, The Netherlands, and carried out in accordance with their guidelines.

Reagents
Biotinylated lectins SNA-I (Sambucus nigra), PNA (Arachis hypogea), and WGA (Triticum vulgaris) were purchased from EY Laboratories Inc., MALII (Maackia amurensis), GSL-I (Griifonia simplicifolia), AAL (Aleuria aurantia), and conjugated streptavidin-phycocerythrin from BD Pharmingen, 7- aminoactinomycin D (AAD) viability dye from eBioscience, NeutrAvidin-Texas Red and DAPI nucleic acid stain from Molecular Probes, Mowiol 4-88 mounting medium from Merck, carboxyfluorescein succinimidyl ester (CFSE) from CellTrace, and PureCol (bovine collagen type I) from Nutacon BV. Poly-L-lysine, purified Neu5Ac, and ManNAc were purchased from Sigma-Aldrich and Clostridium perfringens sialidase and fibronectin from Roche. P-Neu5Ac and the fluorinated P-3Fax-Neu5Ac were synthesized as previously described by others (22, 28).

Cell lines
B16F10 melanoma cells were obtained from and authenticated by American Type Culture Collection (ATCC; CRL-6475) and cultured in minimum essential medium (MEM; Gibco, Invitrogen) containing 5% FBS (Greiner Bio-One), 1% MEM nonessential amino acids (Gibco), 0.15% sodium bicarbonate (Gibco), 1 mmol/L sodium pyruvate (Gibco), 1.5% MEM vitamins (Gibco), 0.5% antibiotic–antimycotic solution (50 U/mL penicillin, 50 µg/mL streptomycin, and 125 ng/mL amphotericin B; PAA). The 9464D neuroblastoma cell line (kindly provided by R. Orentas, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI) was cultured in Dulbecco’s modified Eagle’s medium (Glutamax, Gibco) with 10% FBS, 1% nonessential amino acids, 20 µmol/L 2-mercaptoethanol (Sigma-Aldrich), and 1% antibiotic–antimycotic solution (29). GL261 glioblastoma cells (kindly provided by U. Herrlinger Department of Neurology, University of Bonn, Bonn, Germany) were cultured in Iscove’s modified Dulbecco’s medium (Gibco) supplemented with 10% FBS, 20 µmol/L 2-mercaptoethanol, and 0.5% antibiotic-antimycotic solution. Cells were incubated in a humidified CO2 incubator at 37°C in 5% CO2. All cell lines were initially grown and multiple aliquots were cryopreserved and used within 6 months after resuscitation and tested for mycoplasma using a mycoplasma detection kit (Lonza).

Lectin staining and flow cytometry
After harvesting, cells were washed with carbox-free blocking solution to remove free glycoproteins and incubated for 45 minutes with 5 µg/mL of the respective biotinylated lectin. α2,3-linked sialic acids were detected using MALII, α2,6-linked sialic acids by SNA-I, exposure of terminal (β)-galactose/T antigen by PNA, exposure of terminal (α)-galactose and (α)-GalNAc by GSL-I, (polo) GlcNAc (chitobiose) by WGA, and (α)-fucose by AAL (30–32). Free lectin was removed by washing the cells in carbofree blocking solution, and cell-bound biotinylated lectin was conjugated with 2 µg/mL streptavidin-phycocerythrin for 15 minutes (33). Cell viability was assessed by staining the cells with 7-AAD viability dye. For flow cytometry, cells were washed and resuspended in FACS-buffer (1 × PBS containing 1% FBS and 0.02% sodium azide). Data
Figure 1. P-3F\textsubscript{ax}-Neu5Ac selectively blocks sialylation in B16F10 cells. A, structures of Neu5Ac, P-Neu5Ac, and P-3F\textsubscript{ax}-Neu5Ac. B, representative histograms showing reduced binding of the α\textsubscript{2,3}-sialic acid–recognizing lectin MALII (left) and the α\textsubscript{2,6}-sialic acid–recognizing lectin SNA-I (right) to B16F10 cells treated with PBS or 64 μmol/L P-3F\textsubscript{ax}-Neu5Ac for 3 days. C to H, B16F10 cells were cultured for 3 days in the presence of 0 to 512 μmol/L Neu5Ac, P-Neu5Ac, or P-3F\textsubscript{ax}-Neu5Ac and cell surface sialylation, glucosaminylation, and fucosylation were assessed by flow cytometry using biotinylated lectins conjugated to streptavidin-PE. Presence of α\textsubscript{2,3}-sialylated structures was determined with MALII (C), α\textsubscript{2,6}-sialylation by SNA-I (D), exposure of terminal (β\textsubscript{-})galactose/T antigen by PNA (E), exposure of terminal (α\textsubscript{-})galactose and (α\textsubscript{-})GalNAc by GSL-I (F), (poly)GlcNAc (chitobiose), and sialic acids (partially) by WGA (G) and (α\textsubscript{-})fucose by AAL (H). Fluorescence intensities of the lectins were normalized to the respective untreated cell controls and calculated as percentage sialylation, lectin binding, glucosaminylation, or fucosylation. Data of 3 independent experiments are represented as average values ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
were acquired using a CyAn ADP flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star). Cells stained with only streptavidin–phycoerythrin served as background control. The percentage glycosylation or lectin binding was calculated by normalizing the fluorescence values from the bound lectins to the respective control.

**Inhibitor titration and long-term culture**

To determine the effective dose, B16F10 cells were incubated with 0 to 512 μmol/L of P-3Fα,6-Neu5Ac and as a control Neu5Ac or P-Neu5Ac. Cells were harvested after 3 days of culture, and the glycosylation status was assessed using different biotinylated lectins. In the long-term culture experiment, B16F10 cells were cultured in the presence or absence of 64 μmol/L inhibitor for 28 days. Every third day, condition medium was renewed and sialylation was measured at several time points using the lectins MALII and SNA-I. Viability was assessed during long-term culture with flow cytometry after staining cells with 7-AAD viability dye. To quantify proliferation of treated and untreated B16F10 cells, cell numbers were counted every time before passaging and equal amounts of cells were reseeded. The numbers of treated and untreated cells before passaging were compared to each other.

**Confocal microscopy**

For confocal microscopy, cells were incubated for 3 days in the presence or absence of 64 μmol/L P-3Fα,6-Neu5Ac, harvested, and reseeded on glass slides. Cells were allowed to adhere for 1 hour before they were washed with 1% PBS containing 1% paraformaldehyde. Next, cells were blocked 30 minutes with 1% PBS containing 1% goat serum, washed, and incubated for 1 hour with 2.5 μg/mL biotinylated lectin. After washing, cells were incubated with 5 μg/mL NeutrAvidin-Texas Red for 30 minutes, washed, and stained with 4',6-diamidino-2-phenylindole (DAP) for 10 minutes. Samples were mounted with Mowiol, and images were acquired using an Olympus FV1000 confocal laser scanning microscope (Olympus).

**Recovery of sialylation and sialyltransferase substrate competition assay**

To determine the time of recovery of sialylation, B16F10 cells were treated 3 days with or without P-3Fα,6-Neu5Ac or for 1 hour with 250 μM/mL Clostridium perfringens sialidase in serum-free medium. After extensive washing, cells were reseeded and cultured for 4 days. Cell surface sialylation was assessed every time using the conjugated lectins MALII and SNA-I.

B16F10 cells were cultured for 3 days in medium in the presence or absence of 64 μmol/L P-3Fα,6-Neu5Ac together with increasing amounts of a competitive sugar (0–1000 μmol/L). P-Neu5Ac was used as noninhibiting competitor for P-3Fα,6-Neu5Ac, and ManNac was used to increase the intracellular Neu5Ac production, respectively (34). Cells were collected and stained with MALII to detect α2,3-linked sialic acids, SNA-I for α2,6-linked sialic acids, and furthermore with 7-AAD to confirm viability of the cells. Cells were acquired with flow cytometry, and the fluorescence intensities of bound lectins were quantified.

**Adhesion assay**

Flat-bottom 96-well plates (Maxisorb) were coated with 20 μg/mL type I collagen, 20 μg/mL fibronectin, or 100 μg/mL poly-L-lysine for 1 hour, washed with PBS-T (1× PBS, 0.05% Tween-20) and blocked for 1 hour with PBS-T containing 1% bovine serum albumin. B16F10, GL261, or 9464D cells were treated with 64 μmol/L P-3Fα,6-Neu5Ac for 3 days and labeled with 3 μmol/L CFSE according to the manufacturer’s instructions and resuspended in serum-free medium. A total of 4 × 10⁶ cells were added to the differentially coated wells and allowed to adhere for 45 minutes at 37°C. Nonattached cells were removed by washing the plates carefully 3 times with pre-warmed PBS-T. Subsequently, adherent cells were lysed in lysis buffer (50 mmol/L TRIS-HCl, pH 7.5, 0.1% SDS), and the fluorescence intensity was measured with a Cytofluor II fluorescence multiwell plate reader (PerSeptive Biosystems). Additionally, samples were kept in washing buffer, and images of adherent cells were acquired using a Leica DMIL microscope (Leica).

**Migration assay**

Cell-free gaps were generated using culture inserts (Ibidi GmbH). B16F10 cells, incubated for 3 days with or without 64 μmol/L P-3Fα,6-Neu5Ac, 265 μmol/L P-3Fα,6-Neu5Ac or P-Neu5Ac, were harvested and 4 × 10⁴ cells were added per culture insert chamber, in the presence or absence of the respective sialic acid analogue. After 12 hours, culture inserts were removed; leaving a cell-free gap of approximately 500 μm width and gap closure was followed by microscopy. Images were acquired every 6 hours using a Leica DC300 camera. Images were analyzed and the cell-free area was determined using ImageJ (NIH, Bethesda, MD).

**Tumor mouse model**

B16F10 cells were incubated for 3 days in medium containing PBS, 64 μmol/L P-Neu5Ac, 64 μmol/L P-3Fα,6-Neu5Ac or for 1 hour with 250 μM/mL Clostridium perfringens sialidase in serum-free medium. Cells were washed thoroughly and resuspended in PBS. 0.5 × 10⁶ cells in a total volume of 50 μL were injected s.c. at the right femur. Tumor growth was followed in time, and mice were sacrificed when tumor volume exceeded 1,800 mm³.

**Statistical analysis**

Statistical significance between two groups was determined using the Student’s t test, multiple comparisons were conducted by one-way ANOVA followed by Bonferroni correction and Kaplan–Meier survival curves were analyzed with a log-rank test using Prism 5.03 (GraphPad Software, Inc.), and *P < 0.05* was considered significant (*, *P < 0.05; **, *P < 0.01; ***, *P < 0.001; ****, *P < 0.0001*).
Results

P-3F\textsubscript{ac}-Neu5Ac selectively inhibits sialylation in murine melanoma cells

To show functionality and to determine the effective dosage of P-3F\textsubscript{ac}-Neu5Ac in a murine setting, B16F10 melanoma cells were chosen as a model because of their high expression of sialic acids and metastatic potential (35–37). The cells were incubated for 3 days with increasing concentrations of Neu5Ac, P-Neu5Ac, or the inhibitor P-3F\textsubscript{ac}-Neu5Ac (Fig. 1A), and sialylation was assessed using specific carbohydrate-binding lectins. In line with the findings in human leukemia cells reported by Rillahan and colleagues, 64 μmol/L P-3F\textsubscript{ac}-Neu5Ac was sufficient to reduce α\textsubscript{2,3}-sialylation and α\textsubscript{2,6}-sialylation by more than 90%, as detected by staining with the lectins MALII and SNA-I, respectively (Fig. 1B). Dose–effect experiments revealed that P-3F\textsubscript{ac}-Neu5Ac could significantly reduce expression of α\textsubscript{2,3}-linked sialic acids and α\textsubscript{2,6}-linked sialic acids already at 32 μmol/L, whereas higher concentrations almost completely depleted the cells from surface sialic acids (Fig. 1C and D). Clearly, the blocking effects of P-3F\textsubscript{ac}-Neu5Ac can be ascribed to the attached fluoride and not to other characteristics of the inhibitor, as sialylation was not affected in cells treated with either Neu5Ac or the peracetylated form. Despite blockage of sialylation with P-3F\textsubscript{ac}-Neu5Ac was very efficient, complete loss of α\textsubscript{2,3}-linked sialic acids or α\textsubscript{2,6}-linked sialic acids could not be achieved. At the highest used concentrations of P-3F\textsubscript{ac}-Neu5Ac, a sialoglycan fraction of about 10% remained on the cells. This fraction might represent stable sialoglycans with a slow turnover, sialoglycans that are salvaged from the culture medium, or products of sialyltransferases that are (partially) resistant to the fluorinated sialic acid analogue.

Consistent with decreased sialic acid expression, exposure of terminal galactose residues like the T antigen (Galβ1,3GalNAcαSer/Thr) and terminal N-acetylgalactosamines (GalNAc) increased as reflected by enhanced binding of the lectins PNA and GSL-I (Fig. 1E and F). Almost no binding of PNA or GSL-I was observed to cells cultured with Neu5Ac or P-Neu5Ac. Expression of N-acetylgalactosamines (GlcNAc) and fucose did not vary significantly upon incubation with P-3F\textsubscript{ac}-Neu5Ac, Neu5Ac, or P-Neu5Ac confirming the specificity of P-3F\textsubscript{ac}-Neu5Ac to inhibit STs (Fig. 1G and H). We noted a slight reduction in the binding of WGA to inhibitor-treated cells. This can be explained by the cross-reactivity of lectin to sialic acids rather than by alterations in the expression of GlcNAc (38, 39). Noteworthy, even at the highest concentrations used, tumor cell viability was unaffected by the inhibitor (data not shown).

Next confocal microscopy was used to study the presence of sialic acids on the membrane of inhibitor-treated B16F10 cells. While the α\textsubscript{2,3} sialic acid–binding lectin MALII strongly stained the cell membrane of untreated cells, loss of binding could be observed in cells treated with either Neu5Ac or the peracetylated form. Despite blockage of sialylation with P-3F\textsubscript{ac}-Neu5Ac was very efficient, complete loss of α\textsubscript{2,3}-linked sialic acids or α\textsubscript{2,6}-linked sialic acids could not be achieved. At the highest used concentrations of P-3F\textsubscript{ac}-Neu5Ac, a sialoglycan fraction of about 10% remained on the cells. This fraction might represent stable sialoglycans with a slow turnover, sialoglycans that are salvaged from the culture medium, or products of sialyltransferases that are (partially) resistant to the fluorinated sialic acid analogue.

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![Image](Figure 2. Loss of cell surface sialic acids and exposure of underlying sugar residues. A to L, B16F10 cells were cultured for 3 days in the presence or absence of 64 μmol/L P-3F\textsubscript{ac}-Neu5Ac, plated on cover glasses, and stained with biotinylated lectins and NeutrAvidin-Texas Red (green) followed by nuclear DAPI staining (red). Images of membrane-bound lectins were acquired by confocal microscopy and representative images are presented of untreated cells (A–F) and P-3F\textsubscript{ac}-Neu5Ac–treated cells (G–L). α\textsubscript{2,3}-sialylation was visualized by MALII (A and G), α\textsubscript{2,6}-sialylation by SNA-I (B and H), exposure of terminal β-j galactose/T-antigen by PNA (C and I), exposure of terminal α-galactose and α-galNAc by GSL-I (D and J), (poly)GlcNAc (chitobiose) by WGA (E and K), and α-fucose by AAL (F and L). Cell contours are indicated if required (white stepped lines).)
and fucose residues on the cell membrane remained unchanged after blocking sialylation (Fig. 2E, F, K, and L). Similar results were obtained in other murine and human cancer cell lines (data not shown). These data reveal P-3Fax-Neu5Ac as a potent and specific inhibitor of sialylation in mouse B16F10 melanoma cells.

**P-3Fax-Neu5Ac rapidly affects cell surface sialylation and can be applied for a prolonged time period**

Having confirmed the specific and efficient blocking of sialylation in melanoma cells cultured for 3 days with P-3Fax-Neu5Ac, we next investigated the kinetics of sialylation blockage. Here-to, B16F10 cells were pulsed with 64 or 256 μmol/L P-3Fax-Neu5Ac, and changes in sialylation were measured with MALII and SNA-I in time. At the lower concentration, reduction in α2,3 and α2,6 sialylation could be detected after 6 to 8 hours of incubation (Fig. 3A and B). Strikingly, sialylation was reduced significantly after only 0.5 to 1 hours of incubation and more than halved after 4 hours with the high concentration. Cells kept at 4°C during treatment with a high dose of the inhibitor showed no decrease in sialylation.

Furthermore, we investigated whether P-3Fax-Neu5Ac is suitable to deplete sialic acids permanently in cultured cells. B16F10 cells cultured for 28 days in the presence of 64 μmol/L P-3Fax-Neu5Ac featured permanent loss of cell sialylation.

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**Figure 3.** Fast effect of P-3Fax-Neu5Ac and long-term depletion of sialic acids. A and B, in a pulse chase experiment, B16F10 cells were incubated at 37°C with 64 or 256 μmol/L P-3Fax-Neu5Ac or at 4°C with 512 μmol/L for different time periods, and sialylation was quantified 24 hours after starting the pulse by flow cytometry with MALII (A) and SNA-I (B). Data of minimal 3 independent experiments are displayed as average values ± SEM normalized to control. C and D, long-term culture of B16F10 cells for 28 days in the presence or absence of 64 μmol/L inhibitor. Sialylation was measured using the lectins MALII (C) and SNA-I (D) and is presented as percentage sialylation of untreated cells. E and F, viability and proliferation of B16F10 cells in long-term culture were assessed. Results of 6 independent experiments are presented as percentage viability and percentage proliferation compared to untreated cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
surface sialic acids as measured with the lectins MALII and SNA-I (Fig. 3C and D). However, even after 28 days of culture, complete loss of sialylation could not be achieved. As mentioned above, a stable fraction of about 5% α2,3-linked sialic acids and 20% α2,6-linked sialic acids remained expressed on the cell surface throughout the long-term culture.

The continuous incubation with P-3Fax-Neu5Ac had no apparent effect on cell viability or proliferation of the cells (Fig. 3E and F). These data indicate that P-3Fax-Neu5Ac can block sialylation in a dose-dependent manner after only a few hours of incubation and can be applied over prolonged time periods to block sialylation in culture without causing cellular toxicity.

**P-3F\textsubscript{av}-Neu5Ac causes sustained blockage of sialylation and outcompetes natural sialyltransferase substrates**

To assess longevity of the P-3F\textsubscript{av}-Neu5Ac–mediated block of sialylation, cells were incubated for 3 days with P-Neu5Ac or P-3F\textsubscript{av}-Neu5Ac, extensively washed, and reappearance of cell surface sialylation was determined in time. A control experiment to determine the normal sialoglycan turnover rate in B16F10 cells, recovery of cell surface sialic acids was measured after enzymatic removal using sialidase from *C. perfringens* (40). Efficient loss of α2,3-linked and α2,6-linked sialic acids at the beginning of the experiment (day 0) was confirmed in cells treated with P-3F\textsubscript{av}-Neu5Ac or sialidase. The results showed that within 1 day after enzymatic removal with sialidase, sialic acid expression was fully restored (Fig. 4A and B). For inhibitor-treated cells, 4 days were required for α2,3-linked sialic acids and 2 days for α2,6-linked sialic acids to recover. These data indicate that P-3F\textsubscript{av}-Neu5Ac efficiently inhibits sialylation for prolonged time periods.

There is evidence that not only overexpression of sialyltransferases leads to aberrant sialylation but also tumor cells can exhibit increased metabolic flux rates of the sialic acid synthesis pathway. Here, increased intracellular sialic acid (precursor) levels lead to hypersialylation of...
sialoglycoproteins that are involved in cell adhesion and metastasis (41). Therefore, we investigated whether increased amounts of natural sialyltransferase substrates can neutralize the blocking effect of P-3Fα–Neu5Ac. To this end, B16F10 cells were cultured in the absence or presence of 64 μmol/L P-3Fα–Neu5Ac together with increasing amounts of either RatNeu5Ac or ManNAc. P-Neu5Ac will increase sialic acid levels via the sialic acid salvage pathway and ManNAc can enhance intracellular synthesis of Neu5Ac (42). After 3 days of co-incubation, sialylation of the cells was analyzed. Addition of either P-Neu5Ac or ManNAc to B16F10 cells slightly increased sialylation as expected (Fig. 4C and D). Cancer cells treated with P-3Fα–Neu5Ac had lost expression of sialic acids, and increasing amounts of P-Neu5Ac could not prevent the loss of α2,3-linked sialic acids even at 1,000 μmol/L (Fig. 4C). Addition of 1,000 μmol/L ManNAc could not counteract P-3Fα–Neu5Ac-mediated loss of α2,3-linked or α2,6-linked cell surface sialic acids. Similar results were obtained in cells cultured under the same conditions for 1, 2, or 4 days (data not shown). Altogether, these data show that P-3Fα–Neu5Ac efficiently disrupts sialylation in melanoma cells for a sustained period of time even in the presence of large amounts of competing sialyltransferase substrates.

P-3Fα–Neu5Ac treatment impairs binding to ECM components and migratory capacities

Hypersialylation of adhesion molecules such as integrins has been described to enhance binding of tumor cells to the extracellular matrix (ECM) and to increase their invasive potential (20, 43, 44). Given the high efficacy of P-3Fα–Neu5Ac to block sialylation, we investigated the ability of treated and untreated murine cancer cells to bind ECM components. To this aim, 3 cancer cell lines, B16F10, GL261 (glioblastoma), and 9464D (neuroblastoma), were incubated with or without 64 μmol/L P-3Fα–Neu5Ac for 3 days and their ability to adhere to uncoated wells or wells coated with poly-γ-L-lysine, type I collagen, or fibronectin was tested. While blocking sialylation did not change the capacity of these cells to adhere to uncoated wells, a significant decrease in binding to poly-γ-L-lysine–coated wells was observed (Fig. 5A). Moreover, desialylated cells showed a remarkable impairment in adhesion to type I collagen (Fig. 5A and B). Reduced adhesion to fibronectin–coated plates was detected in GL261 and 9464D cells but not in B16F10 cells.

Ablation of sialylation is correlated with increased motility of cancer cells and promotes metastasis (6, 18, 21). This prompted us to evaluate the potential of P-3Fα–Neu5Ac to alter the migratory capacity of B16F10 cells. Hereto, B16F10 cells treated with or without 64 μmol/L inhibitor were subjected to a gap closure assay. Already after 6 hours of culture, a significant reduction in migration could be detected comparing treated versus untreated cells (Fig. 5C and D). In the course of the experiment, the reduced migratory capacity became even more pronounced as desialylated cells failed to efficiently invade the cell free area. Even after 18 hours of culture, inhibitor-treated cells were incapable of closing the gap. Collectively, these findings indicate that P-3Fα–Neu5Ac can be used to impair cell adhesion to ECM components and cancer cell migration.

Blocking aberrant sialylation in melanoma cells impedes growth in vivo

The interaction with the ECM is essential for tumor cell survival and progression (44). Regarding the potential of P-3Fα–Neu5Ac to impair adhesion ECM components in vitro, we assessed the ability of inhibitor-treated B16F10 cells to grow in vivo. To this aim, B16F10 cells were treated for 3 days with PBS, P-Neu5Ac, P-3Fα–Neu5Ac or 1 hour with sialidase and inoculated subcutaneously into the flank of C57BL/6j mice. Growth of the differentially treated tumor cells and survival of the mice was followed in time. B16F10 cells, treated with the control peracetylated sialic acid analogue, showed similar growth behavior as the PBS-treated cells (Fig. 6A). Cells that were incubated with sialidase showed a slight decrease in growth, whereas the P-3Fα–Neu5Ac–treated cells exhibited significantly delayed growth. The differences in tumor growth were reflected in the survival of the mice (Fig. 6B). While mice that were injected with PBS or P-Neu5Ac–treated cells showed similar median survival times (PBS, 24 days; P-Neu5Ac, 22 days), the median survival time in mice receiving P-3Fα–Neu5Ac–treated cells was almost doubled (40 days). Mice injected with sialidase–treated B16F10 cells showed a slightly increased median survival time (28 days). Note-worthy, metastases to the lungs or livers of the mice could not be detected in any of the treatment groups. Altogether, these data indicate that blocking aberrant sialylation with P-3Fα–Neu5Ac impairs the ability of tumor cells to grow in vivo.

Discussion

Considering the detrimental effects of excessive tumor cells sialylation for patients with cancer, strategies to reduce deranged sialic acid expression are actively explored. In this study, we show that a recently developed fluorinated sialic acid analogue, P-3Fα–Neu5Ac, efficiently inhibits sialylation, adhesion, and migration of murine melanoma cells. While leaving general expression of glycans intact, P-3Fα–Neu5Ac almost completely depleted cell surface sialic acid residues from B16F10 cells for several days. Long-term culture with the inhibitor continuously blocked sialylation without signs of cellular toxicity. Furthermore, our results suggest that P-3Fα–Neu5Ac is a highly specific inhibitor for sialyltransferases that outcompetes endogenous substrates very efficiently. The resulting loss of sialoglycans impaired adhesion of tumor cells to poly-γ-L-lysine, type I collagen, and fibronectin and their migratory capacity. Moreover, here we show...
Figure 5. Blocked sialylation impairs binding to ECM components and impairs migration. A, P-3F<sub>α</sub>-Neu5Ac-treated B16F10, GL261, or 9464D cells were labeled with CFSE and allowed to adhere for 45 minutes to uncoated wells or wells coated with poly-L-lysine, collagen type I, or fibronectin. Nonattached cells were removed by washing the wells, and remnant adhesive cells were lysed and fluorescence signal was measured using a cytofluorometer. Representative images of untreated or P-3F<sub>α</sub>-Neu5Ac–treated cells attached to type I collagen–coated wells are shown (B). C and D, PBS or P-3F<sub>α</sub>-Neu5Ac–treated B16F10 cells were subjected to a gap closure assay using cell culture inserts to generate standardized cell-free areas of 500 μm width. Gap closure was followed in time and images were acquired by microscopy. Representative images are shown for untreated cells (top) and treated cells (bottom; C). Cell-free areas (mm<sup>2</sup>) for each time point were quantified and average values ± SEM of minimal 6 independent experiments are presented (D). *, *P < 0.05; **, *P < 0.01; ***P < 0.001.
for the first time that P-3Fax-Neu5Ac is a potent inhibitor of tumor growth in vivo.

Rillahan and colleagues have described the synthesis of several sialic acid analogues among which P-3Fax-Neu5Ac alone was able to inhibit sialylation in human cancer cell lines at concentrations around 64 μmol/L. In line with their findings, P-3Fax-Neu5Ac blocked α2,3- and α2,6-sialylation efficiently in B16F10 cells at the same concentration. Noteworthy, the effect of this fluorinated sialic acid analogue on α2,8-linked sialic acid expression has not been studied, yet. Synthesis of α2,8-linked sialic acids via α2,8-sialyltransferases relies on α2,3-sialic acid substrates (13). As P-3Fax-Neu5Ac efficiently blocks α2,3-linked sialic acids, α2,8-linked sialic acid expression levels should also be affected. Indeed we observed reduced expression of the α2,8-sialic acid containing disialoganglioside GD2 in neuroblastoma cells upon treatment with P-3Fax-Neu5Ac (data not shown). So far the effectivity of P-3Fax-Neu5Ac has been shown only after several days of culture. Our data show that P-3Fax-Neu5Ac affects melanoma cell sialylation in a dose-dependent manner already after 1 hour of incubation. Long-term culture for 28 days in the presence of the inhibitor permanently reduced sialylation without affecting cellular viability or proliferation. These data rule out that P-3Fax-Neu5Ac (e.g., acetylation or fluorination) causes cellular toxicity or that tumor cells develop resistance toward the inhibitor. These findings are in line with the observation in sialic acid-deficient CHO-Lec2 cells showing that sialylation is not necessary for cell survival in vitro (44).

Up to now, only sialidases have been described to yield cells lacking sialic acids as efficient as P-3Fax-Neu5Ac. Nevertheless, the fast recovery of sialoglycans after enzymatic cleavage and their specificity for certain sialic acid linkages limits the application of sialidases. In this regard, blocking de novo synthesis using P-3Fax-Neu5Ac results in sustained depletion of sialic acids, thus providing a powerful tool to study sialic acids in many biologic processes in human and mouse systems. The sustained blockage of sialylation is potentially due to the accumulation of CMP-ManNAc or P-Neu5Ac supplementation could counteract the P-3Fax-Neu5Ac effects. Our results clearly show that P-3Fax-Neu5Ac inhibits sialylation even in the presence of a large excess (15-fold) of P-Neu5Ac or ManNAc. Noteworthy, blockade of α2,3-sialylation was hardly affected by the competitors, whereas the blockage of α2,6-sialylation could only be partially prevented by high levels of competitor. Again, these findings support...
the idea that P-3Fax-Neu5Ac inhibits α₂,3-sialyltransferases more efficiently than α₂,6-sialyltransferases.

Overexpression of sialyltransferases and the subsequent increase in sialoglycans has been shown to enhance binding to ECM components and migration of cancer cells. Our data now show that P-3Fax-Neu5Ac treatment can be used to block adhesion of cancer cells to collagen type I or fibronectin and to impair their migratory capacity. This effect might be ascribed to α5β1 integrins, as loss of sialylation of these adhesion receptors has previously been shown to reduce binding to type I collagen (20). In contradiction to studies reporting that hyposialylation of α5β1integrins enhances binding to fibronectin, we detected reduced binding to this ECM component after P-3FαNeu5Ac treatment (47, 48). Possibly, other sialoglycoproteins that are affected by blocking global sialylation with P-3Fax-Neu5Ac are involved in binding to fibronectin, too.

Regarding the vital role of hypersialylated structures in cancer progression and migration, blocking sialyltransferases and subsequently sialylation might be of therapeutic benefit (18). We observed that melanoma cells treated with P-3FαNeu5Ac before injection into mice feature delayed growth resulting in increased survival time of the mice. A similar but less pronounced effect was observed in mice that received sialidase-treated cells. This difference between the P-3FαNeu5Ac and the sialidase group can presumably be explained by the different recovery times of sialylglycan expression that we have observed in vitro. While P-3FαNeu5Ac blocked sialylation for 2 to 4 days in vitro, sialoglycan expression was restored within 1 day after sialidase treatment. Most likely, tumor cells recover full sialylation after inoculation in vivo within these periods, too. Therefore, the delayed outgrowth of the melanomas and the prolonged survival of mice that we observed can potentially be ascribed to early events in tumor engraftment that are affected by blocked sialylation. On the basis of our in vitro data, the impairment of hyposialylated adhesion receptors such as integrins to interact with the ECM could contribute to the reduced tumor outgrowth. Although the underlying mechanisms remain elusive, the observed impact of temporarily blocked sialylation on tumor growth and survival suggests that permanent administration of P-3FαNeu5Ac to the tumor could lead to therapeutic effects.

In conclusion, our data show that P-3FαNeu5Ac is a highly efficient inhibitor of aberrant cancer sialylation. The sustained inhibition of sialylation, its potency in the presence of an excess of endogenous competitors, and the functional impact on cell adhesion, migration, and in vivo growth make this novel glycomimetic an attractive tool for anti-cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Büll, M.H. den Brok, G.J. Adema
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Büll, M. Wassink, A.M.A. de Graaf, G.J. Adema
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Büll, M. Wassink, A.M.A. de Graaf, M.H. den Brok, G.J. Adema
Writing, review, and/or revision of the manuscript: C. Büll, F.L. van Delft, M.H. den Brok, G.J. Adema
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Büll, M. Wassink, G.J. Adema
Study supervision: C. Büll, G.J. Adema

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References


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Christian Büll, Thomas J. Boltje, Melissa Wassink, et al.


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