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Improving the Antigenicity of sTn antigen by Modification of Its Sialic Acid Residue for Development of Glycoconjugate Cancer Vaccines

Jian Wu and Zhongwu Guo*

Department of Chemistry, Wayne State University, 5101 Cass Avenue, Detroit, Michigan 48202, USA, zwguo@chem.wayne.edu

Abstract

Sialyl Tn (sTn) antigen is a sialylated disaccharide abundantly expressed by many tumors. To search for effective cancer immunotherapies based on sTn antigen, this work designed and synthesized a series of unnatural *N*-acyl derivatives of sTn and studied their immunological property. For this purpose, an efficient method was developed to synthesize the natural and unnatural forms of sTn antigen and their protein conjugates. The resultant glycoconjugates were used to immunize C57BL/6 mice, and the immune response was assessed by ELISA. Whereas the keyhole limpet hemocyanin (KLH) conjugate of sTn elicited low levels of IgM antibodies, the KLH conjugates of *N*-iso-butanoyl sTn and *N*-phenylacetyl sTn, especially the latter, induced high titers of antigen-specific IgG antibodies, showing a T-cell dependent response which is critical for the antitumor activity. The results suggest that the modified forms of sTn, especially *N*-phenylacetyl sTn, have improved antigenicity and promising immunological properties for being used as cancer vaccines.

Introduction

Many tumors express certain unique carbohydrates termed tumor-associated carbohydrate antigens (TACAs), and the TACAs are closely correlated with cancer cell adhesion and metastasis (1,2). TACAs are also useful molecular targets in the design and development of novel strategies for cancer diagnosis and therapy, such as cancer vaccines (3–9). Among numerous TACAs identified (2–5), sialyl Tn antigen (sTn, Figure 1), an *O*-linked disaccharide [NeuAc α (2→6)GalNAc], is of particular interest. First, sTn is relatively tumor-specific. Even though sTn antigen is richly expressed on a number of tumors, such as breast, prostate, colorectal and ovarian cancer (10–13), it is rarely observed on normal tissues. Second, the expression of sTn has been identified as an independent indicator for poor prognosis of cancer (11–14). Consequently, sTn antigen and relevant mucins that are aberrantly glycosylated with sTn have been extensively investigated for the immunotherapy of cancer (15–20). The protein conjugates of sTn antigen could indeed elicit humoral immune response that showed remarkable specificity for cancer cells (21). The keyhole limpet hemocyanin (KLH) conjugate of sTn antigen (19,20) has been developed and tested as a therapeutic vaccine (Theratope®) for treatment of metastatic breast cancer (22,23). Unfortunately, at the Phase III clinical trial Theratope® failed to meet the endpoints of time-to-disease progression and overall survival rate (23), which was largely because it cannot induce strong T-cell-mediated immune response in patients. In fact, most natural TACAs elicit IgM antibodies rather than IgG antibodies (24), while T-cell dependent IgG response is critical for immunotherapy of cancer (25,26). The

* Corresponding author: tel. 313-577-2557; fax. 313-577-8822.

problem of lacking a T-cell dependent immune response to TACAs has severely hindered further progress of TACA-based cancer vaccines.

To overcome such problems, our group has recently developed a novel strategy for immunotherapy (27), which is based on metabolic engineering of cell surface *N*-acetylneuraminic acid (Neu5Ac) (28,29). The concept is to immunize cancer patients with a synthetic vaccine made of an artificial *N*-acyl derivative of a natural sialyl TACA. After a specific immune response is established, the patients will be treated with correspondingly *N*-acylated D-mannosamine to induce the expression of this artificial antigen on tumor cells in place of the natural TACA. The provoked immune system will then eliminate the bioengineered tumors. As sTn antigen contains a sialic acid residue at its nonreducing end, we anticipated that the new strategy should be applicable to it for the development of useful cancer immunotherapy.

For the new strategy to work, an important condition is an effective vaccine that can induce a T-cell dependant immune response. The other condition is a method that can effectively bioengineer tumor cells (30). The main aim of this work is to study the influence of chemical modification of sialic acid residue of sTn on its antigenicity and identify a derivative that may be used as a vaccine to induce robust T-cell dependent immune responses. For this purpose, we have synthesized the natural and unnatural *N*-acyl derivatives of sTn, linked them to KLH, and studied the immunological properties of the resultant glycoconjugates.

Experimental Procedures

2-Azidoethyl *O*-[methyl 4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-trifluoroacetamido-D-glycero- α -D-galacto-non-2-ulopyranosylonate]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -D-galactopyranoside (**16**)

A mixture of **14** (400 mg, 1.21 mmol), **15** (700 mg, 1.21 mmol) and activated molecular sieves (4 Å, 2.0 g) in anhydrous acetonitrile (5.0 mL) was stirred at rt for 2 h under an atmosphere of argon. After the mixture was cooled to -35°C , NIS (1.1 g, 4.84 mmol) and TfOH (53 μL , 0.61 mmol) were added with stirring. It was kept at -35°C for 30 min and then diluted with DCM (20 mL). The solid material was filtered off and washed with DCM (10 mL). The combined filtrates were washed with aqueous $\text{Na}_2\text{S}_2\text{O}_3$ (20%) and water. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified on a silica gel column (EtOAc/hexane 2:1) to give a mixture of α -/ β -disaccharide (680 mg, 65%, α / β 4:1) as a white foamy solid. $R_f = 0.19$ (EtOAc/hexane 2:1); HR FABMS (m/z): calcd for $\text{C}_{33}\text{H}_{46}\text{F}_3\text{N}_5\text{NaO}_{18}$ ($M + \text{Na}^+$) 880.2688, found 880.2685. This mixture was directly used in the next step of reaction without further purification.

A solution of above product (600 mg, 0.7 mmol) in 65% HOAc/ H_2O (v/v, 6 mL) was stirred at 60°C for 1.5 h. TLC showed the complete consumption of starting material. The mixture was concentrated and co-evaporated with toluene (3×5 mL). The residue was purified by flash column chromatography (DCM/MeOH 18:1) to afford compound **16** (420 mg, 70%) as a white solid ($R_f = 0.27$, DCM/MeOH 12:1) and its β anomer (100 mg, 17%, $R_f = 0.32$, DCM/MeOH 12:1). Compound **16**: $[\alpha]_D -42.4$ (c 1.0, CHCl_3); ^1H NMR (CDCl_3 , 600 MHz): δ 6.72 (d, J 9.6 Hz, 1H), 6.17 (d, J 3.6 Hz, 1H), 5.37-5.26 (m, 2H), 5.21 (dd, J 7.8, 1.8 Hz, 1H), 4.99-4.93 (m, 1H), 4.50 (d, J 8.4 Hz, 1H, H-1), 4.36 (dd, J 12.6, 3.0 Hz, 1H), 4.27 (dd, J 10.8, 2.4 Hz, 1H), 4.10 (dt, J 10.8, 3.6 Hz, 1H), 4.00 (dd, J 12.6, 6.6 Hz, 1H), 3.98-3.93 (m, 2H), 3.82 (s, 3H), 3.79-3.67 (m, 6H), 3.59-3.54 (m, 1H), 3.28 (dt, J 13.2, 3.0 Hz, 1H), 2.91 (s, 1H), 2.63 (dd, J 12.6, 4.2 Hz, 1H, H-3'e), 2.15, 2.14, 2.05, 2.03, 2.01 (5s, $5 \times 3\text{H}$, Ac), 1.96 (t, J 12.6 Hz, 1H, H-3'a). HR FABMS (m/z) calcd for $\text{C}_{30}\text{H}_{42}\text{F}_3\text{N}_5\text{O}_{18}$ ($M + \text{H}^+$) 818.2555, found 818.2552.

***N*-{2-*O*-[[Methyl 4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-trifluoroacetamido-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonate]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl} 4-pentenamide (17)**

A solution of **16** (330 mg, 0.404 mmol) in MeOH (15 mL) was stirred with 10% Pd/C under a H₂ atmosphere at rt for 1 h. After the catalyst was filtered off, to the filtrate that contained glycosyl amine was added 4-pentenoic anhydride. The mixture was stirred at rt overnight. The solution was condensed, and the crude product was purified by flash chromatography (DCM/MeOH 10:1) to afford compound **17** (270 mg, 80%) as a white solid. *R*_f = 0.19 (DCM/MeOH 12:1). [α]_D –39.5 (c 1.0, CHCl₃); ¹H NMR (CDCl₃, 600 MHz): δ 7.08 (d, *J* 9.0 Hz, 1H), 6.99 (d, *J* 5.4 Hz, 1H), 6.41 (s, 1H), 5.86–5.78 (m, 1H, CH₂=CH–), 5.38–5.32 (m, 1H), 5.25 (dd, *J* 7.8, 1.2 Hz, 1H), 5.08 (d, *J* 16.8 Hz, 1H, CH₂=CH–), 5.02 (d, *J* 10.2 Hz, 1H, CH₂=CH–), 4.98 (dt, *J* 11.4, 4.2 Hz, 1H), 4.50 (d, *J* 8.4 Hz, 1H, H-1), 4.39 (dd, *J* 12, 1.8 Hz, 1H), 4.31 (dd, *J* 10.8, 1.8 Hz, 1H), 4.04–3.98 (m, 2H), 3.94 (d, *J* 3.6 Hz, 1H), 3.88–3.74 (m, 7H), 3.70–3.64 (m, 3H), 3.26–3.20 (m, 1H), 2.65 (dd, *J* 12.6, 4.8 Hz, 1H, H-3'e), 2.41–2.36 (m, 2H), 2.33–2.28 (m, 2H), 2.16, 2.15, 2.09, 2.03, 2.02 (5s, 5 $\times$ 3H, Ac), 1.96 (t, *J* 12.6 Hz, 1H, H-3'a). HR FABMS (*m/z*) calcd for C₃₅H₅₀F₃N₃NaO₁₉ (M + Na⁺) 896.2888, found 896.2896.

Compounds 18–21

To a stirred solution of **17** (220 mg, 0.252 mmol) in methanol (10 mL) was added a sodium methoxide solution in methanol (0.1 M, 1.8 mL). After the mixture was stirred at rt for 5 h, it was neutralized with Amberlite IR-120 (H⁺) resin. The resin was filtered off and washed with methanol. The filtrate and washings were combined and concentrated to a small volume (ca. 10 mL), to which was added an aqueous NaOH solution (1 N, 3 mL). After the reaction finished within 24 h, as indicated by TLC, the mixture was neutralized with diluted HCl to pH 7. Then, the reaction mixture was concentrated in vacuum to give the fully deprotected disaccharide, which was directly used in the next step. [α]_D20 –23.2 (c 1.0, MeOH); ¹H NMR (D₂O, 600 MHz): δ 5.86–5.78 (m, 1H, CH₂=CH–), 5.04 (dd, *J* 17.4, 1.8 Hz, 1H, CH₂=CH–), 4.97 (dd, *J* 10.2, 1.8 Hz, 1H, CH₂=CH–), 4.33 (d, *J* 8.4 Hz, 1H, H-1), 3.94–3.76 (m, 6H), 3.74–3.46 (m, 8H), 2.81 (t, *J* 9.6 Hz, 1H), 2.76 (dd, *J* 12.6, 4.8 Hz, 1H, H-3'e), 2.36–2.24 (m, 4H), 1.97 (s, 3H, CH₃CONH), 1.55 (t, *J* 12.0 Hz, 1H, H-3'a). HR FABMS (*m/z*) calcd for C₂₄H₄₀N₃Na₂O₁₄ (M + Na⁺) 640.2306, found 640.2324.

To a solution of above product (40 mg, 0.065 mmol) in MeOH (6 mL) was added an acyl anhydride (Ac₂O 0.1 mL, Pr₂O 0.12 mL, *i*Bu₂O 0.14 mL, or PhAc₂O 100 mg). After the reaction finished within 4 h, as indicated by TLC, the mixture was condensed under reduced pressure. The residue was purified on a Biogel P-2 column with H₂O as the eluent. Fractions containing the expected product were combined and freeze-dried to afford *N*-acyl sTn derivatives **18–21**.

***N*-{2-*O*-[[5-acetamido-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl} 4-pentenamide (18)**

¹H NMR (D₂O, 600 MHz): δ 5.75–5.67 (m, 1H, CH₂=CH–), 4.94 (d, *J* 17.4 Hz, 1H, CH₂=CH–), 4.89 (d, *J* 9.6 Hz, 1H, CH₂=CH–), 4.29 (d, *J* 8.4 Hz, 1H, H-1), 3.83–3.71 (m, 5H), 3.68 (t, *J* 10.2 Hz, 1H), 3.63–3.47 (m, 7H), 3.43 (dd, *J* 9.0, 1.2 Hz, 1H), 3.27–3.16 (m, 2H), 2.58 (dd, *J* 12.0, 4.2 Hz, 1H, H-3'e), 2.24–2.16 (m, 4H), 1.89, 1.88 (2s, 2 $\times$ 3H, CH₃CONH), 1.55 (t, *J* 12.6 Hz, 1H, H-3'a). HR ESIMS (*m/z*) calcd for C₂₆H₄₂N₃Na₂O₁₅ (M + Na⁺) 682.2406, found 682.2407.

***N*-(2-*O*-[5-propanoylamido-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl) 4-pentenamide (19)**

^1H NMR (D_2O , 600MHz): δ 5.75-5.67 (m, 1H, $\text{CH}_2=\text{CH}-$), 4.94 (dd, J 16.8, 1.8 Hz, 1H, $\text{CH}_2=\text{CH}-$), 4.89 (dd, J 10.2, 1.8 Hz, 1H, $\text{CH}_2=\text{CH}-$), 4.29 (d, J 8.4 Hz, 1H, H-1), 3.83-3.71 (m, 5H), 3.68 (t, J 10.2 Hz, 1H), 3.63-3.47 (m, 7H), 3.41 (dd, J 9.0, 1.8 Hz, 1H), 3.27-3.16 (m, 2H), 2.59 (dd, J 12.6, 4.8 Hz, 1H, H-3'e), 2.24-2.16 (m, 4H), 2.15 (q, J 7.8 Hz, 2H, $\text{CH}_3\text{CH}_2\text{CO}$), 1.88 (s, 3H, CH_3CONH) 1.55 (t, J 12.6 Hz, 1H, H-3'a), 0.97 (t, J 7.8 Hz, 3H, $\text{CH}_3\text{CH}_2\text{CO}$). HR ESIMS (m/z) calcd for $\text{C}_{27}\text{H}_{44}\text{N}_3\text{Na}_2\text{O}_{15}$ ($\text{M} + \text{Na}^+$) 696.2562, found 696.2566.

***N*-(2-*O*-[5-(2-methylpropanoylamido)-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl) 4-pentenamide (20)**

^1H NMR (D_2O , 600MHz): δ 5.75-5.67 (m, 1H, $\text{CH}_2=\text{CH}-$), 4.94 (dd, J 16.8, 1.8 Hz, 1H, $\text{CH}_2=\text{CH}-$), 4.89 (dd, J 10.2, 1.8 Hz, 1H, $\text{CH}_2=\text{CH}-$), 4.29 (d, J 9.0 Hz, 1H, H-1), 3.84-3.46 (m, 13H), 3.39 (dd, J 9.0, 1.8 Hz, 1H), 3.27-3.16 (m, 2H), 2.59 (dd, J 12.6, 4.2 Hz, 1H, H-3'e), 2.39 (m, 1H, Me_2CHCO), 2.24-2.16 (m, 4H), 1.88 (s, 3H, CH_3CONH) 1.55 (t, J 12.6 Hz, 1H, H-3'a), 0.97, 0.96 (2d, $2 \times$ 3H, J 6.6 Hz, Me_2CHCO). HR ESIMS (m/z) calcd for $\text{C}_{28}\text{H}_{46}\text{N}_3\text{Na}_2\text{O}_{15}$ ($\text{M} + \text{Na}^+$) 710.2719, found 710.2719.

***N*-(2-*O*-[5-phenylacetamido)-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl) 4-pentenamide (21)**

^1H NMR (D_2O , 600MHz): δ 7.29-7.18 (m, 5H), 5.74-5.66 (m, 1H, $\text{CH}_2=\text{CH}-$), 4.94 (dd, J 17.4, 1.2 Hz, 1H, $\text{CH}_2=\text{CH}-$), 4.89 (dd, J 10.2, 1.2 Hz, 1H, $\text{CH}_2=\text{CH}-$), 4.28 (d, J 8.4 Hz, 1H, H-1), 3.84-3.46 (m, 15H), 3.37 (dd, J 11.4, 6.0 Hz, 1H), 3.26-3.16 (m, 2H), 2.59 (dd, J 12.6, 4.8 Hz, 1H, H-3'e), 2.21-2.16 (m, 4H), 1.88 (s, 3H, CH_3CONH) 1.53 (t, J 12.6 Hz, 1H, H-3'a). HR ESIMS (m/z) calcd for $\text{C}_{32}\text{H}_{46}\text{N}_3\text{Na}_2\text{O}_{15}$ ($\text{M} + \text{Na}^+$) 758.2719, found 758.2710.

Compounds 22–25

Ozone was bubbled into individual solutions of **18–21** (20 mg) in MeOH (10 mL) at -78°C until a blue color appeared. The solutions were kept at -78°C for another 10 min, and then nitrogen was introduced to remove the remaining ozone. After Me_2S (0.5 mL) was added at -78°C , the resultant solutions were allowed to warm to rt over a period of 1 h and stand for another 1 h before it was condensed in vacuum. The crude products were purified on a Biogel P-2 column using distilled water as the eluent to give aldehydes **22–25** as white solids, which were used in the following conjugation reactions without further purification.

***N*-(2-*O*-[5-acetamido-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl) 4-oxo-butanamide (22)**

^1H NMR (D_2O , 600 MHz): δ 4.89 (t, J 6.0 Hz, 1H, $-\text{CH}(\text{OH})_2$), 4.30 (d, J 8.4 Hz, 1H, H-1), 3.44 (d, J 9.0 Hz, 1H), 2.70 (t, J 6.6 Hz, 1H), 2.59 (dd, J 12.6, 4.8 Hz, 1H, H-3'e), 2.50-2.38 (m, 1H), 2.27-2.16 (m, 1H), 1.88 (s, 6H, CH_3CONH), 1.78-1.68 (m, 1H), 1.54 (t, J 12.0 Hz, 1H, H-3'a).

***N*-(2-*O*-[5-propanoylamido-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl) 4-oxo-butanamide (23)**

^1H NMR (D_2O , 600 MHz): δ 5.24 (t, J 6.0 Hz, 1H, $-\text{CH}(\text{OH})_2$), 4.29 (d, J 8.4 Hz, 1H, H-1), 3.41 (d, J 8.4 Hz, 1H), 3.28-3.18 (m, 1H), 2.59 (dd, J 12.6, 4.8 Hz, 1H, H-3'e), 2.50-2.41 (m, 1H), 2.29-2.22 (m, 2H), 2.15 (q, J 7.8 Hz, 2H, $\text{CH}_3\text{CH}_2\text{CO}$), 1.89 (s, 3H, CH_3CONH), 1.78-1.73 (m, 1H), 1.54 (t, J 12.6 Hz, 1H, H-3'a), 0.97 (t, J 7.8 Hz, 3H, $\text{CH}_3\text{CH}_2\text{CO}$).

***N*-(2-*O*-{[5-(2-methylpropanoylamido)-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl} 4-oxo-butanamide (24)**

^1H NMR (D_2O , 600 MHz): δ 5.24 (t, J 3.0 Hz, 1H, $-\text{CH}(\text{OH})_2$), 4.28 (d, J 8.4 Hz, 1H, H-1), 3.39 (d, J 9.0 Hz, 1H), 3.28-3.18 (m, 1H), 2.59 (dd, J 12.6, 4.2 Hz, 1H, H-3'e), 2.50-2.19 (m, 4H), 1.89 (s, 3H, CH_3CONH), 1.80-1.71 (m, 1H), 1.55 (t, J 12.0 Hz, 1H, H-3'a), 0.97, 0.96 (2d, $2 \times 3\text{H}$, J 6.6 Hz, Me_2CHCO).

***N*-(2-*O*-{[5-phenylacetamido)-3,5-dideoxy-*D*-glycero- α -*D*-galacto-non-2-ulopyranosylonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- β -*D*-galactopyranosyl]-ethyl} 4-oxo-butanamide (25)**

^1H NMR (D_2O , 600 MHz): δ 7.30-7.17 (m, 5H), 5.23 (t, J 3.6 Hz, 1H, $-\text{CH}(\text{OH})_2$), 4.27 (d, J 8.4 Hz, 1H, H-1), 3.38 (dd, J 12.0, 6.6 Hz, 1H), 3.24-3.16 (m, 2H), 2.59 (dd, J 12.6, 4.8 Hz, 1H, H-3'e), 2.49-2.40 (m, 1H), 2.34-2.18 (m, 2H), 1.89 (s, 3H, CH_3CONH), 1.80-1.70 (m, 1H), 1.54 (t, J 12.6 Hz, 1H, H-3'a).

General procedure for the coupling between 22–25 and KLH or HAS

A solution of **22**, **23**, **24** or **25** (8 mg), KLH or HSA (6 mg), and NaBH_3CN (6 mg) in 0.1 M NaHCO_3 (0.2 mL, pH 7.5–8.0) was allowed to stand at rt in the dark for 3 days with occasional shaking. The reaction mixture was then loaded onto a Biogel A0.5 column (1 cm $\times$ 15 cm) and eluted with 0.1 M PBS buffer ($I = 0.1$, pH = 7.8). The fractions containing the glycoconjugate, characterized by BCA assay for proteins and by the Svennerholm method for sialic acid, were combined and dialyzed against distilled water for 2 days. It was then lyophilized to give a white powder of the expected glycoconjugates **4–11** (5–6 mg).

Analysis of the carbohydrate loading levels of the glycoconjugates (31,32)

After the solution of an exactly weighed sample of the glycoconjugate (0.5 mg) in distilled water (1.0 mL) was mixed with the resorcinol reagent (2.0 mL), the mixture was heated in a boiling water bath for 30 min. It was cooled to rt, and to this mixture was then added an extraction solution (1-butanol acetate and 1-butanol, 85:15 v/v, 3.0 mL). The mixture was shaken vigorously before it was allowed to stand still for *ca.* 10 min, allowing the organic layer to separate well from the inorganic layer. The organic layer was transferred to a 1.0 cm cuvette, and its absorbance at 580 nm was determined by an UV-vis spectrometer, using a blank organic solution as the control. The sialic acid content of the glycoconjugate is determined against a calibration curve created with the standard NeuNAcyl (Acyl = Ac, Pr, iBu, PhAc) solutions analyzed under the same condition. The carbohydrate loading of each glycoconjugate was calculated according to the equation shown below.

$$N - \text{Acyl sTn loading}(\%) = \frac{\text{amount of sialic acid (mg) in the sample}}{\text{weight of the glycoconjugate sample (mg)}} \times \frac{\text{molecular weight of sTn}}{\text{molecular weight of sialic acid}} \times 100\%$$

Immunization of mice

Five female C57BL/6 mice at the age of 8 weeks (Jackson Laboratories, Bar Harbor, ME) were immunized for each sTn-KLH conjugate. Immunizations were intraperitoneal with the glycoconjugate containing 2 μg of carbohydrate in 200 μL of saline mixed with 200 μL of MPL/TDM Ribi adjuvant (Sigma Chemical, St. Louis, MO) following the manufacturer's protocol. The mice were boosted with identical immunizations on days 15 and 22 following the initial immunization. The mice were bled by tail vein prior to the initial immunization on day 0 and after immunization on day 14 and day 31. Bleed was clotted to obtain sera, which were stored at -80°C .

Protocols for ELISA analysis

ELISA plates were first treated with 100 μ l solution of KLH or the HSA conjugate of sTn or sTn derivatives (1 μ g/ml 0.1 M bicarbonate buffer) overnight at 4 °C, followed by washing with PBS. Pooled sera from each group of mice were diluted 1:300 to 1:72900 in serial half-log dilutions in PBS with 0.02% azide, and incubated overnight in the coated ELISA plates (100 μ l/well). The plates were then washed and incubated with 1:1000 dilution of alkaline phosphatase linked anti- κ , anti-IgM or anti-IgG2a antibodies or with 1:2000 dilution of anti-IgG1 or anti-IgG3 antibodies (Southern Biotechnology, Buckingham, AL) for 1 h at rt. Plates were washed and developed with PNPP substrate for colorimetric readout using a BioRad 550 plate reader at 405 nM wavelength.

For titer analysis, optical density (OD) values were plotted against dilution values, and a best-fit line was obtained. The equation of this line was employed to calculate the dilution value at which an OD of 0.5 was achieved, and antibody titer was calculated at the inverse of this dilution value.

Results and Discussion

In the design of sTn analogs, we were focused on the modification of sialic acid residue, because previous studies have demonstrated that cells can be readily bioengineered to express unnatural sialic acids following the uptake of *N*-acyl mannosamines as biosynthetic precursors (30,33–36). The sTn derivatives synthesized and studied in this work were *N*-propionyl sTn (sTnNPr, **1**), *N*-*iso*-butanoyl sTn (sTnNiBu, **2**) and *N*-phenylacetyl sTn (sTnNPhAc, **3**) (Figure 2). The same *N*-acyl derivatives of sialic acid proved to be more antigenic than Neu5Ac itself (37); therefore, we expected that **1**, **2** and **3** would be better antigens than sTn. On the other hand, it has been reported that *N*-propionyl and *N*-phenylacetyl mannosamines could efficiently glycoengineer tumor cells (30,31). It is thus reasonable to expect the feasibility to achieve glycoengineered modification of sTn on tumors by these functional groups, which is necessary for the new immunotherapeutic strategy. Consequently, if certain glycoconjugates designed above show desired immunological properties, they will have great potentials for being used as vaccines in the exploration of new cancer immunotherapies.

To form effective vaccines, sTn and its *N*-acyl derivatives was coupled to a carrier protein. We chose KLH for this purpose, since KLH has shown useful immunological properties (15,38). Alternatively, to avoid the detection of antibodies specific for KLH in ELISA, human serum album (HSA) was employed as the carrier protein of the capture reagents. The antigens and carrier proteins were coupled together by a convenient and efficient method based on the pentenoyl group (39). An additional advantage of this coupling method is that the linker itself does not cause immune response (40).

The synthesis of natural sTn antigen, its unnatural *N*-acyl derivatives and their protein conjugates is outlined in Scheme 1. The glycosyl acceptor **14** was prepared from D-galactosamine (**12**) after a series of transformations including conversion of **12** into a 2-azidoethanol glycoside **13** followed by complete *O*-deacetylation and then selective protection of the 3,4-hydroxyl groups as an acetonide. A major issue in this synthetic scheme is the sialylation reaction, which is one of the most difficult glycosylation reactions in carbohydrate chemistry. We used Boons and coworkers' method (41) to achieve this transformation, in which an *N*-trifluoroacetyl derivative of neuraminic acid was employed as the glycosyl donor to offer the desired α -glycoside. The trifluoroacetyl group as a protecting group was easily removable to facilitate N-deprotection and subsequent modification with various acyl groups, offering a highly convergent route to the target structures. Thus, after the required sialyl donor **15** was prepared from Neu5Ac following a literature procedure (41), it was used to glycosidate **14** using *N*-iodosuccinamide (NIS) and triflic acid (TfOH) as the promoters. This reaction afforded

the disaccharide in a good yield (65%) and a reasonable stereoselectivity (α/β : 4/1), but the two anomers were almost inseparable by column chromatography. However, we found that after the acetonide was removed, the diol products were easily separated to afford pure **16**. The α -configuration of its sialic acid was determined by the unique, downfield shifted NMR signal of H-3e (δ 2.63, compared to δ 2.35 of β -linked sialic acid), which was further confirmed by structural analysis of subsequent products. Compound **16** was then converted into *N*-acyl derivatives of sTn and linked to the carrier proteins.

The pentenoyl group used to couple carbohydrate antigens to carrier proteins was introduced upon selective reduction of the azido group to form a primary amine and then selective acylation of the amino group with pentenoic anhydride. The product **17** was a common intermediate in the synthesis of sTn and its *N*-acyl derivatives. The *N*-trifluoroacetyl group and all *O*-acetyl groups were selectively removed by treatments with a methanolic solution of NaOMe and a methanolic aqueous solution of NaOH, in the presence of an *N*-pentenoyl group. To the exposed amino group were then regioselectively introduced acyl groups in methanol by reactions with acetic, propionic, *iso*-butyric and phenylacetic anhydrides to afford **18–21**, respectively. These products were purified on a Biogel P2 column. Their identity and purity were supported by NMR and HR MS. At this stage, the α -configuration of sialic acid was further confirmed by a downfield shifted H-3e NMR signal (δ 2.59) compared to the β -anomer (δ < 2.40).

Finally, sTn and its derivatives were coupled to the carrier proteins by a two-step procedure (39). First, **18–21** were treated with ozone in methanol to selectively oxidize their carbon-carbon double bond and produce hydrated aldehydes **22–25** that were purified on a Biogel P2 column. The ^1H NMR spectra of **22–25** clearly showed the hydrated aldehyde signal at δ 5.2, whereas the signals of protons linked to carbon-carbon double bond disappeared completely. Next, **22–25** were coupled to the carrier proteins, KLH and HSA, by reductive amination carried out in a 0.1 M NaHCO_3 buffer in the presence of NaBH_3CN . The sTn derivatives **22–25** were employed in excess, and the unreacted **22–25** were readily separated from the glycoconjugate products on a Biogel A0.5 column. Fractions containing the glycoconjugate, which was the first eluted component, were combined and dialyzed against distilled water. Freeze drying of the aqueous solution afforded glycoconjugates **4–11** as white fluffy solids.

The sialic acid contents of resultant glycoconjugates **4–11** were analyzed by Svennerholm method (32). The contents were converted into carbohydrate loading levels according to the equation presented in the Experimental Section. As shown in Table 1, the coupling reactions yielded glycoconjugates containing 8–13% (w/w) of carbohydrates. The loading level of HSA conjugates was lightly higher than that of KLH conjugates, which might be determined by the property of carrier proteins. Nevertheless, the level of antigen loading in these glycoconjugates is typical for glycoconjugate vaccines.

The immunological properties of glycoconjugates **4–7** as vaccines were studied in C57BL/6 mice. For immunizations, each glycoconjugate containing ca. 2 μg of carbohydrates mixed with MPL/TDM Ribi adjuvant (42) was injected intraperitoneally in mice. These mice were then boosted with the same glycoconjugate on day 15 and day 22 following the initial immunization. On day 14 and day 31, animal sera were withdrawn and pooled with respect to the individual group and date. The titer of total antigen-specific antibody or a specific antibody isotype including IgM, IgG1, IgG2a and IgG3 of the pooled sera was assessed by ELISA.

In ELISA, plate wells were first coated with a derivatized sTn-HSA conjugate or KLH alone as the capture antigen and then treated with the pooled day -1, day 14 or day 31 sera of five immunized mice in a glycoconjugate group. Pooling the sera of immunized mice in each group gave a mean serum antibody concentration. Antibodies captured by the coating antigen on plates were detected with goat anti-mouse kappa light chain specific and horseradish

peroxidase (HRP) conjugated antibody, which would reflect about 95% of the total antibody response (26). Using sTn-HSA conjugates as the capture antigens allowed us to detect antibodies that were specific for the modified sTn component of KLH conjugates without detection of anti-KLH antibodies. The assays using KLH as the capture antigen served as a positive control, since KLH is well established as a strong immunogen to elicit high titers of antibodies. The sera obtained after the primary immunization, namely obtained on day 14, gave low ELISA titers, so only the ELISA data of sera obtained on day 31 are presented and discussed in detail here.

Figure 3A shows the total antibody titers with the optical density (OD) plotting against pooled sera dilutions. Figure 3B gives the normalized titer analysis data (Experimental Section) which can quantify more accurately the antigenicity of the glycoconjugates. Although all of the tested glycoconjugates 4–7 were immunogenic, sTnNPhAc gave obviously the highest level of antigen-specific antibodies, and the natural sTn antigen gave the lowest concentration of sTn-specific antibodies. There was essentially no binding of pre-immune serum to various sTn-HSA conjugates.

Figure 4 shows the titers of different isotypes of antibodies. All glycoconjugates produced relatively equivalent concentrations of IgM antibodies (panel A), but significant difference was observed with IgG responses. Panel B reveals that the tested glycoconjugates induced low levels of IgG1 antibody, but sTnNPhAc and sTnNiBu induced significant amount of IgG2a antibodies (panel C). IgG3 titers (panel D) showed a hierarchy similar to that of antigenicity in Figure 3B.

Finally, we also examined the reactivity of antisera from modified sTn-immunized mice to the HSA conjugate of natural sTn (Figure 5). The antisera of sTnNPr and sTnNiBu showed low binding titers, indicating some cross reactivity to sTn. Since sTn is relatively tumor-specific, this cross reactivity may not have a major impact on normal tissues. In fact, under certain conditions the synthetic vaccines with improved immunological properties, combined with the cross reactivity of their immune responses to native sTn antigen as well as the unique presentation of sTn on tumor cells, may be useful for cancer immunotherapy. However, sTnNPr-KLH and sTnNiBu-KLH are definitely not the ideal vaccines for our new strategy of cancer immunotherapy, due to the cross reactivity and relatively poor antigenicity. In contrast, the reactivity of sTnNPhAc sera to sTn was too low to be detected. This result suggests that the immune response induced by sTnNPhAc was specific.

Above ELISA data suggest that chemical modifications of sTn antigen could improve its antigenicity, which is consistent with the conclusions about other TACAs (27,37,40,43,44). Moreover, sTnNPhAc proved to be the most antigenic among the sTn derivatives investigated. Most importantly, though the natural sTn antigen induced exclusively IgM antibodies (Figures 3 and 4), sTnNPhAc induced significant titers of IgG antibodies, which indicates a T-cell dependent immune response, such as antibody isotype switching, antibody affinity maturation, immunological memory, and the induction of cell-mediated cytotoxicity (6,25,26). The switch to T-cell dependent immune response is important for the antitumor activity of cancer vaccines (45). The reasons for the antigenicity improvement of modified forms of sTn antigen and their capability to stimulate T-cell dependent immune responses are not clear, but we think that the significant difference of the structure and chemical properties of sTnNPhAc from native sTn, especially the increased hydrophobicity of the former, may play a critical role. For instance, this may facilitate the recognition and binding of sTnNPhAc by the immune system and immune cells. ELISA data of the reactivity of antisera obtained from mice immunized with modified sTn-KLH conjugates to native sTn antigen revealed that sTnNPhAc sera had essentially no cross reaction. This may well suggest that when being used as cancer vaccines the sTnNPhAc conjugate would cause no or relatively low autoimmune reactions. This result

may be explained by the significantly structural difference between the phenylacetyl group in sTnNPhAc and the acetyl group in native sTn. The high antigenicity of sTnNPhAc-KLH combined with its relatively low potential to cause autoimmune reactions makes it an excellent vaccine.

In summary, this paper has established an expedient method for synthesizing N-modified derivatives of sTn antigen and their protein conjugates. After careful analysis of the immunological properties of the synthetic glycoconjugates, we identified sTnNPhAc-KLH as a promising candidate for being developed into a functional cancer vaccine, not only because sTnNPhAc is highly antigenic, but also because it can induce IgG immune responses that are critical for the antitumor applications (25,26). An effective glycoconjugate vaccine based on sTnNPhAc combined with the fact that cancer cell surface Neu5Ac can be readily and effectively engineered by *N*-phenylacetyl-D-mannosamine may provide a solution to the problem of immune tolerance to sTn and may be used for the immunotherapy of cancer which expresses high levels of sTn antigen, such as breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.
Structures of *N*-modified sTn antigens and the glycoconjugate vaccines studied

Figure 3.

Antigen-specific total antibody contents in sera analyzed by ELISA. (A) Each line represents the antibody level in serum pooled from a group of five mice. Anti-sTn and anti-derivatized sTn antibody levels were obtained from mice immunized with glycoconjugates 4–7. Anti-KLH antibody level was obtained from mice immunized with sTn, and anti-sTn antibody level of pre-immune serum was obtained from the same group and used as the negative control (equivalent results were obtained from other groups). Error bars are smaller than the symbol width. (B) Normalized ELISA data of total antibodies. A best-fit line was obtained by plotting OD values against dilution values, and the equation of this line was used to calculate the dilution value at which an OD of 0.5 was achieved.

Figure 4. Titer analysis of different isotypes of antigen-specific antibodies determined by ELISA assay. Each represents the titer in pooled serum obtained on day 31 after primary and booster immunizations from five replicate animals.

Figure 5.

Titer analysis of the cross reactivity determined by ELISA. Each bar represents total anti-sTn reactivity in pooled sera from five replicate animals immunized with a derivatized sTn-KLH conjugate.

Scheme 1.

Reagents and conditions: (a) NaOMe, MeOH, 99%; (b) dimethoxypropane, p-TsOH, DMF, 65 °C, 90%; (c) NIS, TfOH, MS 4Å, MeCN, -35 °C, 65%; (d) 60% HOAc, 65 °C, 87%; (e) 10% Pd/C, H₂, MeOH, 1 h; then 4-pentenoic anhydride, overnight, 80%; (f) 0.1 N MeONa/MeOH; then 1 N NaOH, 24 h; finally various anhydrides, rt, >95%; (g) O₃, MeOH, -70 °C, 0.5 h; then Me₂S, to rt, 2 h, 85–90%; (h) KLH or HSA, NaBH₃CN, 0.1 N NaHCO₃, rt in dark, 3 days.

Table 1

Antigen loading level of the glycoconjugates

sample	KLH conjugates			HSA conjugates		
	4	5	6	7	8	11
loading (%)	8	10	9	10	11	13
					14	10