Enhancement of the Immunogenicity of Synthetic Carbohydrate Vaccines by Chemical Modifications of STn Antigen†

Fan Yang,‡,† Xiu-Jing Zheng,‡,† Chang-Xin Huo,‡ Yue Wang,‡ Ye Zhang,‡,* and Xin-Shan Ye‡,*

‡State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences
§School of Basic Medical Sciences
Peking University, Xue Yuan Rd 38, Beijing 100191, China

ABSTRACT: The abnormal glycans expressed on the surface of tumor cells, known as tumor-associated carbohydrate antigens, increase the chance to develop carbohydrate-based anticancer vaccines. However, carbohydrate antigens pose certain difficulties, and the major drawback is their weak immunogenicity. To tackle this problem, numerous structurally modified STn antigens were designed and synthesized in this work. These synthetic antigens were screened in vitro by using competitive ELISA method, and the antigens with positive response were conjugated to the protein carrier for vaccination. The vaccination results on mice showed that some fluorine-containing modifications on the STn antigen can significantly increase the anti-STn IgG titers and improve the ratios of anti-STn IgG/IgM. The antisera can recognize the tumor cells expressing the native STn antigen.

RESULTS AND DISCUSSION

STn antigen (1 in Figure 1), an O-linked disaccharide [NeuAc-(2→6)GalNAc], was chosen for this purpose, because the expression of STn on normal cells is limited but is abundant on a wide range of tumors such as breast, prostate, pancreas, colorectal, lung, gastric, and ovarian cancers, which makes STn a relatively specific tumor-associated antigen for vaccination.25 The development of tumor can be efficiently affected by anti-STn antibodies,26,27 which makes STn-based vaccine more promising.
Another fact is that when Theratope, a STn-KLH (keyhole limpet hemocyanin) conjugation vaccine, was subjected to phase III clinical trial, the result was not so encouraging because it did not induce strong T-cell-mediated immune response in patients, and only modest clinical efficacy was achieved when patients were treated in conjugation with hormone therapy.25 One possible reason for the partial failure of Theratope could be insufficient enhancement of STn immunogenicity. We hope that the suitable modifications28 on STn antigen by a chemical approach will enhance the immunogenicity of STn.

Natural STn is linked to a serine or threonine site of protein through an $R$-O-glycosidic bond,25 and many efforts on the synthesis of STn have been reported.24,29-31 In our case, allyl alcohol was used as the surrogate of the amino acid residues with the $R$-O-glycosidic bond retained (2 in Figure 1). The O-allyl group also functioned as a protecting group for the anomeric OH and facilitated the consequent conjugation with proteins.32

The diversity of structural modifications needs to be augmented to find better modified antigens (Figure 1). The two N-acetyl groups of STn could be conveniently replaced by many other acyl groups (3-6, 10-12, 15-19, 23-24, 27-30, 34-36). Besides these acyl-substituted modifications, the N-acetyl group was also replaced by azido or free amino groups (13, 14, 25, 26). In addition, the carboxylic group of STn was changed to other groups such as hydroxamic acid (37), which is a weaker acid and plays important roles in some inhibitor designs.35 Other groups such as $\text{CH}_2\text{OH}$ (38) and $\text{COOMe}$ (39) were also possible substitutes for the $\text{-COOH}$. Since the specificity of the recognition of antibodies with different glycoforms is still controversial,34 altering the glycoform in the antigen could also provide a chance to enhance the immunogenicity. Therefore, the galactosamine moiety (GalNAc) of STn antigen was replaced by similar glycoforms such as galactose (Gal) (40) and glucosamine (GlcNAc) (41). Furthermore, the anomic isomer NeuAc$\beta$-(2-6)GalNAc (42) of STn was also designed for the immunological test.

Fluorinated compounds are frequently used in medicinal chemistry and have led to a large number of highly effective drugs.35 However, the fluorine substitution strategy has not been examined for improving the immunogenicity of vaccines.36,37 The fluorinated modifications might provide hopeful opportunities for vaccine development, for fluoro-substituted structures may be highly immunogenic as a result of the absence of fluorine in most organisms, whereas the similar atom radius and lipophilicity compared to the hydrogen atom may satisfy the structural and functional requirements. So the fluorinated modifications (7-9, 20-22, 31-33) of STn were designed.

Starting from $D$-N-acetylgalactosamine (43), the allyl group was introduced as a tether to produce the allyl-linked compound 44 via the Fischer glycosylation38 (Figure 2). To improve the solubility of the compound 44 toward glycosylation reactions, 3-O-benzoyl (Bz) protected acceptor 46 was prepared via the intermediate 45 by functional group transformations. It should be noted that the 3-O-Bz group was able to migrate under basic conditions when using base to neutralize the acid employed for

![Figure 1. Structures of native antigen and structure-modified antigens.](image-url)
the deprotection of the benzylidene group, and this problem was avoided by using the resin-based acid without neutralization. The sialylation reactions in acetonitrile\(^{39,40}\) gave products in low yields (\(\sim 20\%\)) due to the low solubility of acceptors \(46\) and \(53\). However, when tetrahydrofuran (THF) was used as solvent instead of acetonitrile, the sialylation efficiency was greatly improved, providing disaccharides \(48\), \(52\), and \(54\) in good isolated yields.

Deprotection of the \(O\)-acyl groups in disaccharide \(48\) by MeONa/MeOH gave compound \(39\) in \(95\%\) isolated yield, which was further saponified under \(1\) N NaOH aqueous conditions producing compound \(2\). Similarly, the \(\beta\)-isomer \(42\) was obtained from the corresponding precursor (Supporting Information). Treatment of the methyl ester \(39\) with NH\(_2\)OH \(^{41}\) yielded the hydroxamic acid derivative \(37\), whereas the reduction of \(39\) provided the primary alcohol derivative \(38\). As a result of the activation of methyl ester in compound \(39\) by its anomeric atom, convenient conditions such as NaBH\(_4\)/MeOH provided the reduction product in \(94\%\) yield.

With the stronger basic conditions (\(2\) N NaOH, \(90\) °C), the two N-acetyl groups in compound \(2\) were hydrolyzed to yield the key intermediate \(50\) in which the free amino functionalities were subjected to derivations. For the modifications of different acyl groups, the acylation conditions differed. Generally, simple fatty acylations were performed with the corresponding anhydrides, but this protocol did not work well for halogen-containing acylations, and the conversion efficiency dropped dramatically despite various solvents and bases employed. Therefore, for the monochloride-containing acylation (preparation of \(34\)), it proved to be efficient to use the corresponding carboxylic acid in the presence of the coupling reagent \(O\)-(benzotriazol-1-yl)-\(N,N,N',N'\)-tetramethyluronium hexafluorophosphate (HBTU), whereas for other halogen-containing acylations, the methyl esters of carboxylic acids were used to obtain acylated products by the condensation of methyl esters and amine \(50\). Thus, many diacylated ST\(_n\) derivatives (\(27\)–\(36\)) were prepared by the modifications of different acyl groups. To prepare the monoacylated ST\(_n\) derivatives, disaccharide \(52\), formed in high yield and with better stereoselectivity by the
coupling reaction of trifluoroacetyl (TFA)-substituted sialic acid donor 51,42,43, and acceptor 46, was treated with bases at RT, providing the selectively deprotected product 14 with the N-acetyl group in GalNAc moiety intact (Figure 2). The free amino group of 14 was then modified by different acyl groups to produce various monoacylated products 3–12 and 15. Azido-substituted product 13 was also prepared via the modified diazo-transfer procedure.44 On the other hand, after deprotection of the ester groups in disaccharide 54, the azido functionality was reduced to a free amino group, yielding compound 26, which was also subjected to modifications in the same way as mentioned above to obtain the monomodified STn derivatives 16–25. In addition, the glycoform-replaced analogues, NeuAcα-(2−6)Gal (40) and NeuAcα-(2−6)GlcNAc (41), were prepared in the similar manner as the synthesis of compound 2. To sum up, 40 modified STn derivatives were obtained.

With various modified antigens in hand, a preliminary screening was performed based on an in vitro test. It is necessary to check whether the modified antigens can be recognized by the antibodies elicited by the native STn antigen. So the competitive enzyme-linked immunosorbent assay (ELISA) was carried out 13,15,16,43 in which the antibodies induced by the native STn-based vaccine were employed and the unmodified type STn-BSA (bovine serum albumin) as coating antigen. After in vitro screening (Supporting Information), there were a total of 28 synthetic antigens having comparable antibody-recognition abilities, and these compounds were allowed to conjugate with the carrier protein for further in vivo testing.

Compound 2 and 28 modified carbohydrate antigens needed to be conjugated to protein carriers for vaccination and analysis (Figure 3). All of the conjugates except for 14-KLH (or 14-BSA) were prepared through a two-step procedure.32 That is, each STn derivative was treated with ozone in methanol to selectively oxidize the carbon−carbon double bond of the anomeric O-allyl group, producing an aldehyde intermediate; the aldehyde intermediate was subsequently coupled with carrier proteins including KHL and BSA by reductive amination in the aqueous buffer solutions, accomplishing the synthesis of glycoconjugates. For the conjugation of antigen 14, the above procedure was not applicable, so another approach was adopted. Thus, 9-KLH (or 9-BSA) conjugate was treated with basic aqueous buffer (pH = 10) solution to afford 14-KLH (or 14-BSA), which was detected by 19F NMR. The epitope ratios of glycoconjugates (including carbohydrate-KLH and carbohydrate-BSA) were determined by estimating sialic acid content using the resorcinol method described by Svennerholm 46 and protein content by BCA assay.47

Figure 3. Preparation of the glycoconjugates.
Table 1. Immunological Results after Vaccination with Synthetic Carbohydrate Conjugates

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG/IgM</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG/IgM</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG/IgM</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG/IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-KLH</td>
<td>910</td>
<td>&lt;1000</td>
<td>100</td>
<td>256</td>
<td>5763</td>
<td>8.70</td>
<td>20</td>
<td>20</td>
<td>100</td>
<td>30</td>
<td>50</td>
<td>0.60</td>
</tr>
<tr>
<td>20-KLH</td>
<td>11000</td>
<td>&lt;1000</td>
<td>100</td>
<td>185</td>
<td>2581</td>
<td>71.81</td>
<td>40</td>
<td>40</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>0.50</td>
</tr>
<tr>
<td>21-KLH</td>
<td>1266</td>
<td>&lt;1000</td>
<td>100</td>
<td>150</td>
<td>9236</td>
<td>16.30</td>
<td>123</td>
<td>123</td>
<td>100</td>
<td>150</td>
<td>150</td>
<td>1.00</td>
</tr>
<tr>
<td>31-KLH</td>
<td>20000</td>
<td>&lt;1000</td>
<td>100</td>
<td>276</td>
<td>1804</td>
<td>153.08</td>
<td>279</td>
<td>279</td>
<td>100</td>
<td>799</td>
<td>799</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ELISA titer anti-STn after vaccination and ratio of IgG/IgM after third and fourth</th>
<th>ELISA titer anti-modified-STn</th>
</tr>
</thead>
<tbody>
<tr>
<td>after second</td>
<td>after third</td>
</tr>
<tr>
<td>after fourth</td>
<td>after third</td>
</tr>
<tr>
<td>2-KLH</td>
<td>910</td>
</tr>
<tr>
<td>20-KLH</td>
<td>11000</td>
</tr>
<tr>
<td>21-KLH</td>
<td>1266</td>
</tr>
<tr>
<td>31-KLH</td>
<td>20000</td>
</tr>
</tbody>
</table>

*50 μg of STn was used, much weaker binding was observed. Not only IgG titers but also the ratios of IgG/IgM were increased by several folds. These two values are both regarded as key marks for tolerance-breaking in vaccines and will benefit long-term immunity memory. Our experiments also demonstrated the antisera reacted strongly with the STn-positive tumor cells, implying the potential as anti-cancer vaccines. By avoiding the MOE technology, the risk in modification of sialic acid on normal cells would be eliminated. The strategy based on antigen modifications with fluoric or other suitable substitutions might find applications in the development of carbohydrate-based and peptide-based vaccines.

**METHODS**

**General Sialylation Procedure.** Typically, a mixture of glycosyl acceptor (1.07 g, 2.93 mmol, 1.00 equiv), donor (20.06 g, 3.38 mmol, 1.15 equiv), and activated 4 A molecular sieves (0.8 g) in dry THF (40 mL) under a nitrogen atmosphere was stirred for 0.5 h at RT before it was cooled to −72 °C. Trimethylsilyl trifluoromethanesulfonate (TMSOTf) (70 μL, 0.36 mmol, 0.11 equiv) was then added to the mixture in three batches. The reaction mixture was stirred for 2−4 h until TLC analysis indicated that the reaction was completed. Triethylamine (1.0 mL) was added to the mixture. The precipitate was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (petroleum ether/acetone 1.5/1) to afford the corresponding disaccharides (48, 49, and 50) as white foams. Yield: 84%; α/β = 1/1.2.

**General Procedure for Acylation with Fluoro-Containing Substituents.** To a solution of the disaccharide (14, 26, or 50, 10−30 mg, 0.02−0.06 mmol) in methanol (5.0 mL) were added methyl fluoro containing ester (2.0 mL) and triethylamine (1.0 mL) under nitrogen atmosphere. The mixture was stirred at RT to 50 °C for 1−4 h until TLC analysis indicated that the reaction was complete. The solvent was removed under reduced pressure. The residue was purified by C-18 reverse-phase column chromatography (H₂O or H₂O/MeOH as eluent) and Biogel P-2 column chromatography (H₂O as eluent) to yield the products.

**Competitive Inhibition ELISA Assay.** An ELISA plate was coated with 100 μL of STn-BSA (including 0.02 μg of STn) overnight at 4 °C (0.1 M bicarbonate buffer). After being washed three times with PBST, microwells were blocked with BSA. The anti-STn antibody (1:4000 dilution) was mixed with various concentrations of native-STn or modified-STn antigens for 1 h at 37 °C. Then the mixture was added to the microwells and incubated for 1 h at 37 °C. The plate was washed and incubated with a 1:5000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG for 1 h at 37 °C. The plate was washed, developed with o-phenylenediamine (OPD) substrate in the dark for
15 min, terminated with 2 M H₂SO₄ and then read at 490 nm. Percentage inhibition was calculated as the difference in absorbance between the uninhibited and inhibited serum as in eq 1. Every concentration was triplicate wells. The procedure of competitive inhibition ELISA assay of 40, 41, and 2 was performed as the same mentioned above (except antibodies were sera obtained from mice and the second antibody was horseradish peroxidase conjugated goat anti-mouse IgG). The competitive ELISA assays were conducted separately twice.

% inhibition of binding = \frac{A_{\text{without}} - A_{\text{with}}}{A_{\text{without}}} \times 100 \quad (eq 1)

where \(A_{\text{without}}\) is the absorbance of serum without inhibitor and \(A_{\text{with}}\) is the absorbance of serum with inhibitor.

**Preparation of the Glycoconjugates.** A solution of aldehyde-containing disaccharides (5 mg) prepared from ozonization of the allyl-containing disaccharides, KLH (5 mg), and NaBH₃CN (5 mg) in PBS (0.4 mL, 0.1 M, pH = 7.6) was gently shaken in the dark for 12–16 h at RT and then dialyzed against PBS at 4°C (molecular weight cutoff value 14,000 Da, 6 changes of PBS). The epitope ratios of the glycoconjugates (including carbohydrate-KLH and carbohydrate-BSA) were determined by estimating sialic acid content using the resorcinol method described by Svennerholm and protein content by BCA assay.

**Immunization of Mice.** Groups of six mice (female pathogen-free BALB/c, age 6–8 weeks, from Department of Laboratory Animal Science, Peking University Health Science Center) were immunized four times at 2-week intervals with STn-KLH or modified-STn-KLH glycoconjugates (each containing 2 μg of carbohydrate in PBS). The vaccines were administered intraperitoneally. Mice were bled prior to the initial vaccination, 13 days after the second and the third vaccinations, and 14 days after the fourth vaccination. Blood was clotted to obtain sera, which were stored at −80°C.

**Serological Assays.** The total antigen-specific antibody titers of the sera were assessed by means of ELISA. An ELISA plate was coated with 100 μL of STn-BSA (including 0.02 μg of STn) overnight at 4°C (0.1 M bicarbonate buffer, pH = 9.6). After being washed three times with PBST, microwells were blocked with BSA. After the plate was washed, serially diluted sera were added to microwells (100 μL/well) and incubated with a 1:5000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific) (Southern Biotechnology Associates, Inc., Birmingham, AL) for 1 h at 37°C. The plate was washed, developed with o-phenylenediamine (OPD) substrate in the dark for 15 min, terminated with 2 M H₂SO₄, and then read at 490 nm. The antibody titer was defined as the highest dilution showing an absorbance of 0.1, after subtracting background. Meanwhile, the antibody titers (sera from 13 days after the third vaccination) were determined by ELISA, with the plate coated by the corresponding modified-STn-BSA conjugates instead.

**Flow Cytometry.** LS-C (STn positive) and LS-B (STn negative) human colon cancer cells were obtained from Dr. Steven H. Itzkowitz at Mount Sinai School of Medicine. Single-cell suspensions of 5 × 10⁵ cells/tube were washed with 3% fetal calf serum (FCS) in PBS and incubated with 25 μL of 1:20 diluted test sera (sera from preimmunization and 14 days after the fourth vaccination) for 30 min on ice. After two washes with 3% FCS in PBS, 25 μL of 1:15-diluted goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific) labeled with FITC (Southern Biotechnology Associates, Inc., Birmingham, AL) was added, and the mixture was incubated for 30 min on ice. After a final wash, fixed with 1% formaldehyde and assayed using a FACScan (Becton Dickinson).

**ASSOCIATED CONTENT**

**Supporting Information.** Full experimental information including methods and results of compound preparation, methods and results on immunity, and copies of NMR spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.
These authors contributed equally to this work.

**ACKNOWLEDGMENT**

We thank Dr. Steven H. Itzkowitz for his generously providing us with the LS-C cell line. This work was financially supported by the National Natural Science Foundation of China (90713010 and 20732001).

**DEDICATION**

Dedicated to Professor Henry N. C. Wong on the occasion of his 60th birthday.

**REFERENCES**


