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Research Article

Synthesis of Azido-Globo H Analogs for Immunogenicity Evaluation

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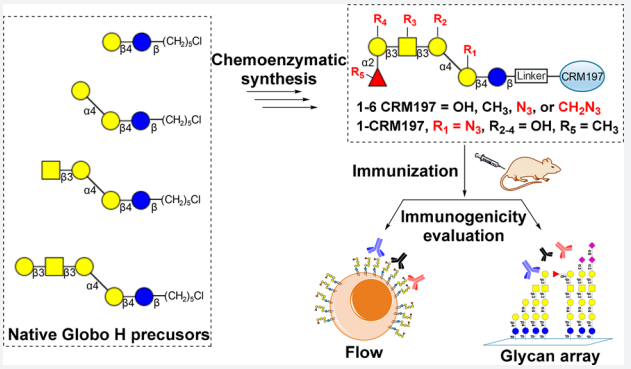
ABSTRACT: Globo H (GH) is a tumor-associated carbohydrate antigen (TACA), and GH conjugations have been evaluated as potential cancer vaccines. However, like all carbohydrate-based vaccines, low immunogenicity is a major issue. Modifications of the TACA increase its immunogenicity, but the systemic modification on GH is challenging and the synthesis is cumbersome. In this study, we synthesized several azido-GH analogs for evaluation, using galactose oxidase to selectively oxidize C6-OH of the terminal galactose or *N*-acetylgalactosamine on lactose, Gb3, Gb4, and SSEA3 into C6 aldehyde, which was then transformed chemically to the azido group. The azido-derivatives were further glycosylated to azido-GH analogs by glycosyltransferases coupled with sugar nucleotide regeneration. These azido-GH analogs and native GH were conjugated to diphtheria toxoid cross-reactive material CRM197 for vaccination with C34 adjuvant in mice. Glycan array analysis of antisera indicated that the azido-GH glycoconjugate with azide at Gal-C6 of Lac (**1-CRM197**) elicited the highest antibody response not only to GH, SSEA3, and SSEA4, which share the common SSEA3 epitope, but also to MCF-7 cancer cells, which express these Globo-series glycans.



INTRODUCTION

Tumor-associated carbohydrate antigens (TACAs) are overexpressed in many types of cancer cells and associated with tumor progression.[1−5] They are considered as potential vaccine targets due to their specific expression on cancer cells. However, TACAs alone are less immunogenic and can hardly induce IgG antibodies and T-cell dependent responses. To overcome this problem, TACA vaccines have been formulated with different strategies, such as conjugation to a carrier,[6] coadministration with adjuvant,[7] construction of multivalent antigens on a carrier,[8,9] and conjugation to immunologic ligands.[10−12] The success of these strategies has led to the development of carbohydrate-based vaccines that induce antibodies to recognize the unique glycan moiety on cancer cells, and some of these vaccines have entered clinical development.[13−15]

The globo-series glycosphingolipids (GSLs), including SSEA3 (Gb5), SSEA4, and Globo H (GH). GSLs are overexpressed in many human cancer cells. Staining with monoclonal antibodies MBr1 and VK9 showed that GH is expressed on colon, ovarian, gastric, pancreatic, endometrial, lung, prostate, breast cancer cells, etc., and breast cancer stem cells.[16−19] The biosynthetic pathway to globo-series GSLs starts with β1,3-galactosyltransferase V (β3GalT5), the key enzyme involved in the synthesis of SSEA3, which is then converted to Globo H and SSEA4. Knockdown of β3GalT5 was found to inhibit cancer cell survival and promote cancer cell apoptosis.[20] Also, the release of GH ceramide from cancer cells promotes angiogenesis and immunosuppression.[21,22] The specific expression of GH in cancer cells and its correlation with cancer progression make GH an ideal target for anticancer vaccine development. Unfortunately, like other TACAs, GH antigen alone does not elicit T cell-dependent immune response, so it is necessary to conjugate GH to a carrier protein for it to induce T cell-dependent immunity. Our group has recently demonstrated that GH conjugated to diphtheria toxin mutant CRM197 (GH-CRM197) can be used as an effective vaccine.[19] When the conjugate is combined with C34 adjuvant, which is designed to enhance immune response and class switch, the vaccine is able to elicit a strong antibody response to GH as well as the related Globo-series antigens SSEA3 and SSEA4.[19] The success of that GH-CRM197 study complemented the development of GH/KLH/QS21 (adaglox-ad simolenin, OBI-822) vaccine, which is currently in global phase 3 trials for the treatment of triple-negative breast cancer (NCT03562637).[23,24]

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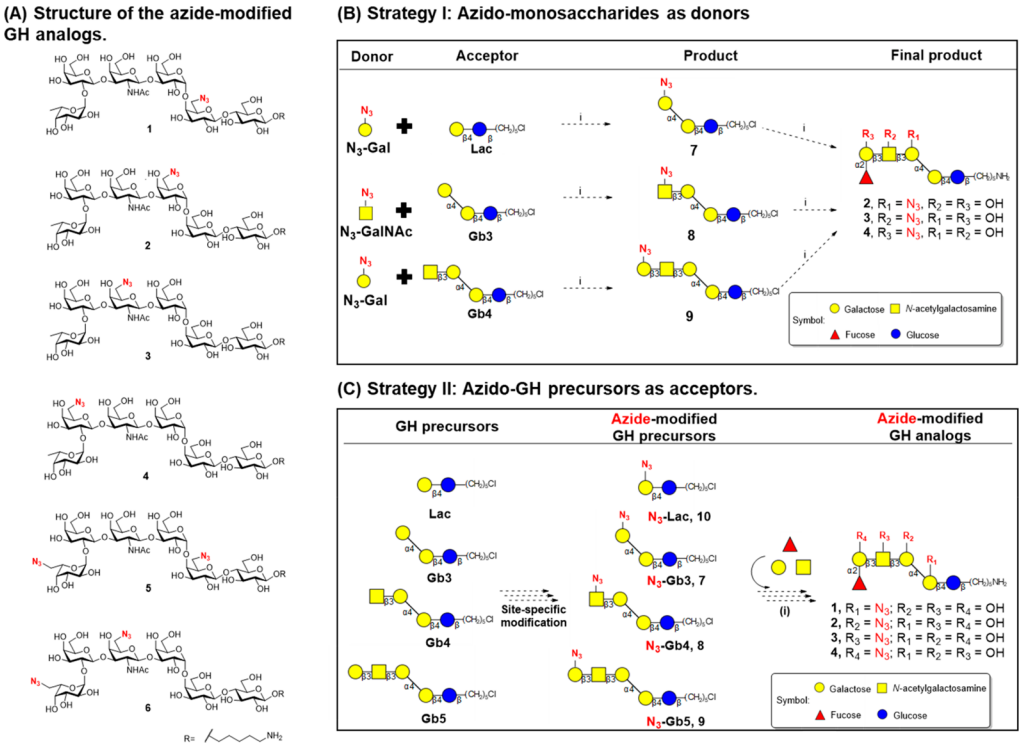

Figure 1. Targeted GH analogs and the synthesis strategy of azido-GH analogs **1**−**6**. (A) Structure of the azide-modified GH analogs. (B) Strategy I: Azido-monosaccharides as donors. (C) Strategy II: Azido-GH precursors as acceptors. Monoazido modification at different sugar moieties of GH (**1**−**4**) and diazido modification on C6-fucose and the nonreducing end C6-Lac (**5**) or Gb4 (**6**). (i) Glycosyltransferases and sugar-nucleotide regeneration system.

To increase the immunogenicity of TACAs-based vaccines, many studies focus on modification of TACAs to induce cross-reactive antibodies that recognize native TACAs.[25,26] The early study focused on modification of the *N*-acetyl group on the sialic acid of GM3-KLH into phenylacetyl GM3-KLH and produced stronger immunogenicity and T-cell-dependent immunity.[27] Later, the same modification on STn-KLH also produced a stronger immune response than native STn-KLH.[28,29] However, in these studies, the antibodies induced by these antigen-modified TACAs did not cross-react well with native TACAs. On the other hand, modification of the *N*-acetyl group to *N*-propionamide on the sialic acid of GM3-KLH vaccine or to *N*-fluoroacetyl group of STn-KLH, TF-CRM197, and GH-CRM197 vaccines induced stronger IgM and IgG antibody responses than their native glycoconjugate vaccines did. Besides, antisera from mice immunized with these TACA-modified vaccines were cross-reactive to native TACA-antigens and could recognize the corresponding cancer cells.[30−34] Moreover, our group also developed GH analogs with azido group at the reducing or nonreducing end of GH. Compared to native GH-CRM197 conjugate, the azido-GH glycoconjugates elicited stronger immune responses,[35] and the antisera from mice immunized with such vaccines recognized and eliminated cancer cells by complement-dependent cytotoxicity. Because the immune system has never encountered the azido group so the azido-GH conjugate elicits a strong immune response to this nonself azido-GH antigen and the closely related GH. Although previous study identified azido-modified GH is more immunogenic, only modification on the reducing and nonducing end of GH were evaluated. To perform the systematic site-specific modification of individual sugar moieties of GH and identify the best hydroxyl group for azide replacement are very challenging and have not been accomplished.

In this study, monoazido-GH analogs with the azido group introduced to the nonreducing end C6 of Gal or GalNAc on lactose (Lac, **1**), Gb3 (**2**), Gb4 (**3**), and SSEA3 (**4**) were first synthesized. We also synthesized diazido-GH analogs containing the azide at C6 of fucose and the nonreducing end C6 of Gal on Lac (**5**) or C6 of GalNAc on Gb4 (**6**) (Figure 1). Galactose oxidase was used to selectively oxidize the glycan acceptors at their terminal Gal or GalNAc to 6-aldehyde, which was transformed into 6-azide derivatives by chemical conversion. The 6-azide derivatives were used as substrates for glycosyltransferases to synthesize azido-GH analogs **1**−**6**, which were then conjugated to CRM197 for immunization with C34 adjuvant in Balb/C mice. Analysis of the antisera showed that the azido-GH glycoconjugate **1-CRM197** and the diazido-GH analog glycoconjugate **5-CRM197** elicited stronger IgG antibody response to native GH, SSEA3, and SSEA4 antigens than **GH-CRM197**. Moreover, **1-CRM197** induced antibodies that can recognize not only GH on the glycan array but also the highly GH expressing cancer cells, MCF-7. Overall, this study demonstrated an efficient approach to the synthesis of various azido-GH. With this method, azido-GHs were synthesized for systematic evaluation of their immunogenicity. Through conjugation and immunization, **1-CRM197** was identified to elicit the most robust IgG antibody response to globo-series antigens (GH, SSEA3, and SSEA4).

RESULTS AND DISCUSSION

Synthesis of Building Blocks for the Preparation of Azido-GH Analogs. In previous studies, both chemical and enzymatic methods were successful in the synthesis of GH

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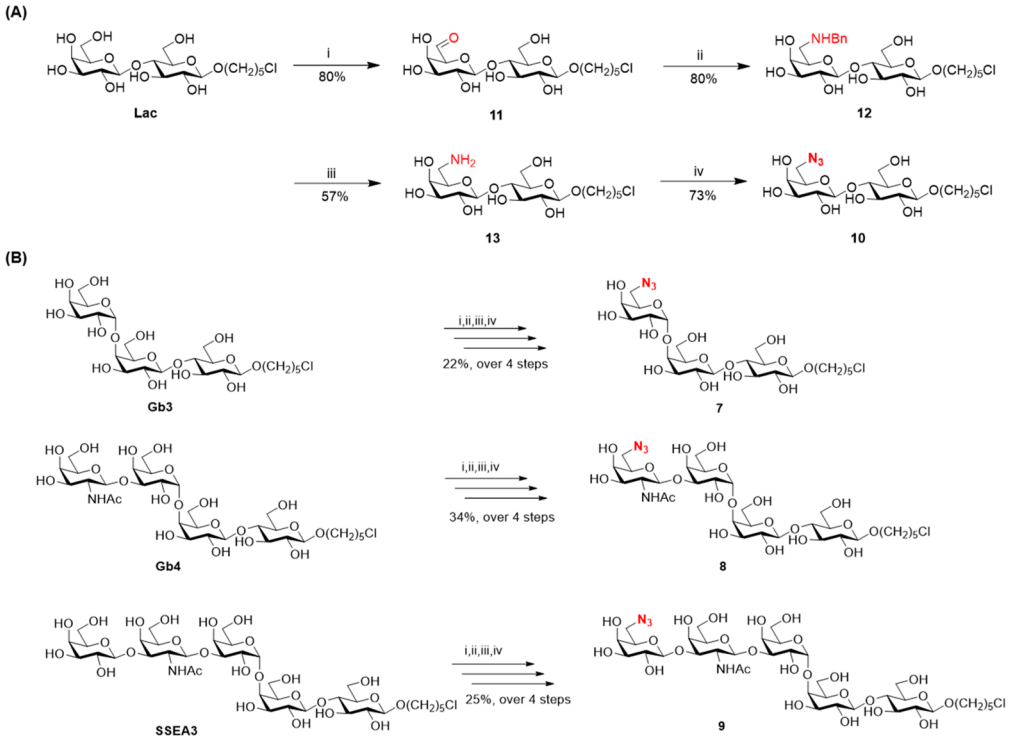
Scheme 1. Site-Specific Synthesis of Azido-GH Precursors. (A) Synthesis of Azide-Modified Lac. (B) Synthesis of Azide-Modified Gb3, Gb4, and SSEA3[a].



[a]Reagents and conditions: (i) galactose oxidase, peroxidase, catalase, and H_2O; (ii) $BnNH_2$, $NaBH_3CN$, and MeOH; (iii) NIS and MeOH; (iv) TfN_3, $CuSO_4$, K_2CO_3, CH_2Cl_2, MeOH, and H_2O.

antigen.[36−42] Programmable one-pot strategy was also developed for the synthesis of GH in excellent yield (17.8% yield over 7 steps,[43] 58.1% yield over 6 steps[44]). However, to synthesize azido-GH analogs, chemical or programmable one-pot method require building blocks with azide functionality and different protecting strategy. Also, the electron-withdrawing characteristics of azide may affect 1,2-*cis*-glycosylation in the preparation of Gb3 building block.[45] On the other hand, enzymatic synthesis of GH coupled with sugar nucleotide regeneration (SNR) was demonstrated to be simpler and practical for large-scale process (Scheme S1).[42] Therefore, we first attempted to synthesize azido-GH analogs with enzymatic strategy, using azido-monosaccharides **N_3-Gal** and **N_3-GalNAc** as donors and **Lac**, **Gb3**, and **Gb4** as acceptors (Figure 1B).

To prepare the acceptors, we followed previous enzymatic strategy and SNR[46] to synthesize **Lac** into **Gb3**, which was further elongated into **Gb4** (Scheme S1). Then, **Gal** and **GalNAc** were modified into **N_3-Gal**[47] and **N_3-GalNAc**[48] (in overall 48% and 8% yield, respectively) and used them as donors for enzymatic synthesis of azido-GH derivatives (Scheme S2). However, **N_3-Gb3** (**7**) was not able to be synthesized by using **N_3-Gal** as donor and **Lac** as acceptor by LgtC and SNR. Moreover, **N_3-Gb4** (**8**) could not be synthesized from **Gb3** acceptor and **N_3-GalNAc** donor by MalE-LgtD and SNR. In the SNR system, Gal was first converted to Gal-1-phosphate by galactokinase (GalK) and then to UDP-Gal by UDP-galactose pyrophosphorylase (AtUSP). **N_3-Gal** has been known as not an effective substrate for GalK, probably due to the loss of required hydrogen bonding at C6 for galactokinase.[47] Previous study indicated that **N_3-GalNAc** was well-tolerated by the SNR enzymes (such as NahK and GlmU).[49] However, we did not further investigate the specificity of the enzymes in the SNR system and the glycosyltransferases, as this will require more effort to find a possible method for enzymatic incorporation of the azido-donors to the acceptors.

To overcome the problem of enzymatic glycosylation using azido-sugar as donor, we decided to directly modify the nonreducing end Gal or GalNAc of native **Lac**, **Gb3**, **Gb4**, and **SSEA3** to **N_3-Lac** (**10**), **N_3-Gb3** (**7**), **N_3-Gb4** (**8**), and **N_3-SSEA3** (**9**), respectively. Then, azido-GH precursors **7**−**10** were used as acceptors to synthesize azido-GH analogs (Figure 1C). In this strategy, native monosaccharides instead of azido-monosaccharides were used as donors for enzymatic glycosylation coupled with SNR. To install the azido group at C6 on terminal Gal or GalNAc of GH precursors by chemical synthesis is time-consuming due to tedious protection and deprotection steps. Moreover, the presence of many primary alcohols on GH precursors makes it difficult to selectively install the azido group at C6 of the nonreducing end. To overcome these issues, we took advantage of galactose oxidase, which was known to catalyze the oxidation of C6 hydroxyl group on the terminal D-Gal or D-GalNAc of oligosaccharide to the corresponding aldehyde with high regioselectivity.[50−52] As expected, the hydroxyl group at the nonreducing end C6 of Gal on **Lac** was oxidized to aldehyde **11** in hydrate form in D_2O, and aldehyde **11** was directly converted to benzylamine through reductive amination without purification to get compound **12** (Scheme 1A). When the benzyl group was deprotected under hydrogenation to afford the free amine, the chloromethyl group in the linker was converted to the methyl group during hydrogenation. The undesired linker cannot be used for conjugation to the carrier protein. Thus, to retain the integrity of linker at reducing end,

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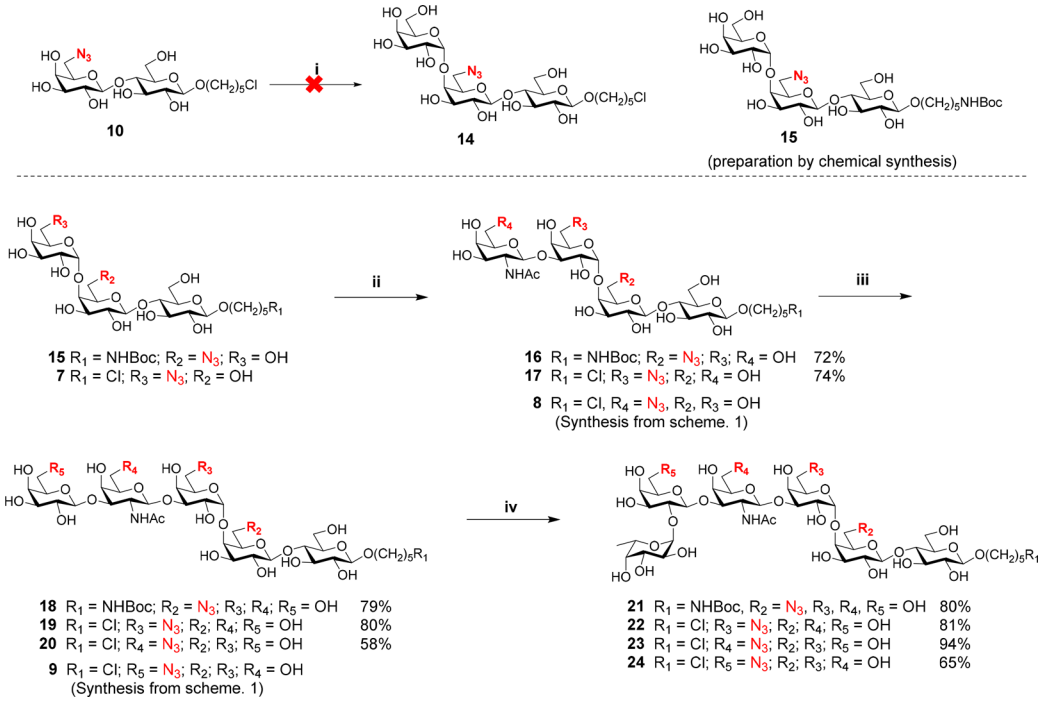
Scheme 2. Enzymatic Glycosylation of Azido-GH Precursors to Azido-GH Analogs[a]



[a]Reagents and conditions: (i) Gal, LgtC, GalK, AtUSP, PK, PPA, and buffer containing Tris-HCl, $MgCl_2$, PEP, UTP, and ATP; (ii) GalNAc, MalE-LgtD, NahK, GlmU, PK, PPA, and buffer containing Tris-HCl, $MgCl_2$, PEP, UTP, and ATP; (iii) Gal, MalE-LgtD, GalK, AtUSP, PK, PPA, and buffer containing Tris-HCl, $MgCl_2$, PEP, UTP, and ATP; (iv) Fucose, FutC, FKP, PK, PPA, and buffer containing Tris-HCl, $MgCl_2$, PEP, UTP, and ATP.

NIS was used to reduce the benzylamine into free amine **13**,[53] which then was converted to azide to afford azido-Lac **10** in 27% yield over four steps. Using this efficient chemoenzymatic synthesis, azido group was able to be selectively installed at the nonreducing C6 of Gal on the Lac moiety.

Then, the synthetic strategy was expanded to other GH precursors, including **Gb3**, **Gb4**, and **SSEA3**, which can be synthesized by previous reported method (Figure S1).[42] All these GH precursors can be oxidized by galactose oxidase to the corresponding aldehyde in their hydrate forms, which then were converted to azide at the C6 of terminal Gal or GalNAc (Scheme 1B). With this method, azido group was installed at the nonreducing end C6 of Gal or GalNAc on **Gb3**, **Gb4**, and **SSEA3** to obtain compounds **7**, **8**, and **9** in 22%, 34%, and 25% yield over four steps, respectively (Schemes S3−S6).

Elongation of Azido-GH Precursors to Azido-GH Analogs for Glycoconjugation. To synthesize azido-GH analogs from azido-GH precursors, the enzymatic method[42] and SNR as described above were used to elongate N_3−Lac **10** to Gb3 analog **14** (Scheme 2A). However, **compound 10** was not able to perform as a substrate in this enzymatic method. Chemical synthesis was the used to prepare Gb3 analog **15** from compound **S10**, which was synthesized by previously reported method (Scheme S7).[35] Gb3 analogs **15** and **7** were elongated to Gb4 analogs **16** and **17** in 72% and 74% yield, respectively, using GalNAc as donor, MalE-LgtD as glycosyltransferase, and UDP-GalNAc regeneration, which includes *N*-acetylhexosamine kinase (NahK), *N*-acetyl glucosamine-1-phosphate uridyltransferase (GlmU), pyruvate kinase (PK), and pyrophosphatase (PPA) (Scheme 2B). Gb4 analogs **16**, **17**, and **8** were elongated into SSEA3 analogs **18**, **19**, and **20** in 79%, 80%, and 58% yield, respectively, using Gal as donor, MalE-LgtD as glycosyltransferase, and UDP-Gal regeneration, which includes GalK, AtUSP, PK, and PPA. Finally, GH analogs **21**, **22**, **23**, and **24** were obtained from SSEA3 analogs **18**, **19**, **20**, and **9** in 80%, 81%, 94%, and 65% yield, respectively, using fucose as donor, α1,2-fucosyltransferase (FutC) as glycosyltransferase, and GDP-Fuc regeneration, which includes bifunctional fucokinase (FKP), PK, and PPA (Schemes S8−S10).

With the chemoenzymatic strategy described above, GH precursors were selectively oxidized by galactose oxidase and converted into azido-GH precursors, which were used as acceptors for glycosyltransferases and SNR. Most of these precursors (**7**, **8**, and **9**) were efficiently elongated into azido-GH analogs with site-specific azide incorporation. Only compound **10** was not elongated by LgtC, so compound **15** was chemically synthesized as acceptor for enzymatic elongation to form azido-GH analogs by glycosyltransferase and SNR. Thus, by selective installation of azide on GH precursors, various azido-GH analogs were obtained for vaccine preparation.

Preparation of GH and Azido-GH Glycoconjugates. To synthesize azido-GH glycoconjugates, NHBoc protecting group at the linker of azido-GH **21** was deprotected under acid treatment to afford azido-GH **1** (Scheme S11). The chloride group at the linker of azido-GH analogs **22**, **23**, and **24** was converted into amine by reaction with ammonium hydroxide to get azido-GH analogs **2**, **3**, and **4** in quant. yield (Scheme S12). In order to conjugate these azido-GH analogs to the carrier protein, azido-GH analogs **1**−**4** were reacted with *p*-nitrophenyl adipate linker to afford the corresponding half esters **S18**−**S21** in 61−71% yield (Scheme S13). Next, native GH and azido-GH analogs half-esters **S18**−**S21** were

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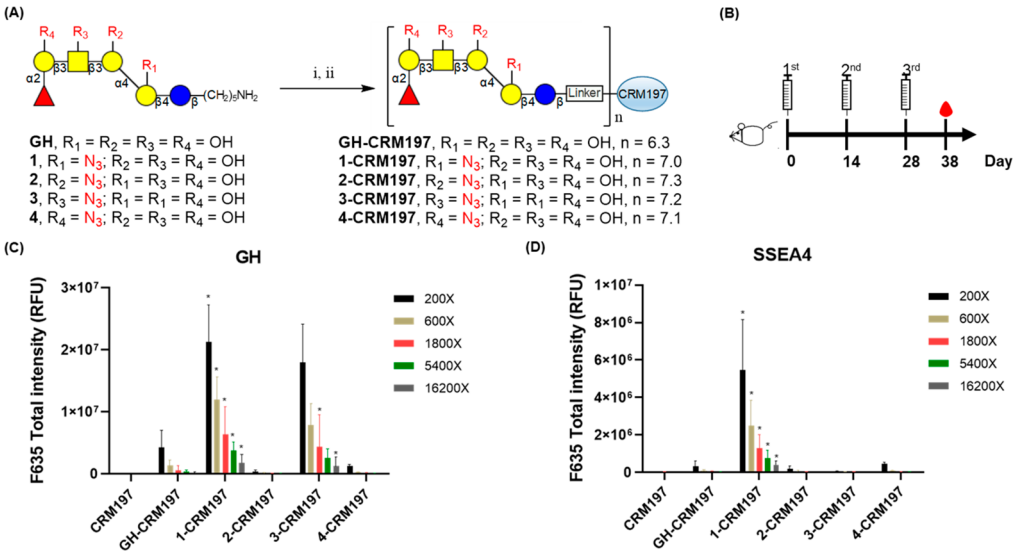

Figure 2. Preparation of GH analog vaccines and immunization study. (A) Synthesis of GH and azido-GH analogs vaccines. (B) GH analog vaccine immunization schedule. (C) IgG level against GH on glycan array. (D) IgG level against SSEA4 on glycan array. Mice number $n = 5$. Each serum was analyzed by glycan array in 5 different dilution folds (200×: black bar; 600×: brown bar; 1800×: red bar; 5400×: green bar; 16200×: gray bar). Secondary antibody used in the array study was Alexa Fluor 647 conjugated goat anti-mouse IgG antibody. Data are means ± SEM (standard error of the mean). Each vaccine was compared with **GH-CRM197** and the comparisons of signal were performed by Mann−Whitney *U* test (unpaired). $*P < 0.05$. RFU: relative fluorescence unit. Reagents and conditions: (i) *p*-nitrophenyl adipate linker, TEA, and DMF; (ii) CRM197 and sodium phosphate buffer (pH 7.9).

conjugated to CRM197 in sodium phosphate buffer (pH 7.9) to obtain GH and azido-GH glycoconjugates **GH-CRM197**, **1-CRM197**, **2-CRM197**, **3-CRM197**, and **4-CRM197** (Figure 2A). The number of azido-GH analogs incorporated into CRM197 was analyzed by MALDI-TOF and SDS-PAGE (Table S1 and Figure S1), and the conjugates were kept around 6.3−7.3 to avoid variation in immunization study.

Glycan Array Analysis of Antisera from Mice Immunized with Azido-GH Glycoconjugates. To study the immunogenicity of azido-GH glycoconjugates, both native GH and azido-GH glycoconjugates were immunized to female Balb/c mice ($n = 5$) with α-galactosylceramide analog C34 as adjuvant, which was shown to induce a class switch from IgM to IgG and a stronger immune response than QS-21 in previous GH vaccine studies.[19] Mice received three shots at two-week intervals between each shot, and each shot contained 2 μg of carbohydrate antigen and 2 μg of C34 (Figure 2B). Mice sera were collected 10 days after the final shot and characterized by a glycan array coated with globo-series glycans, azido-GH analogs **1**−**6**, and other tumor-associated carbohydrate antigens (Table S2). We focused on the analysis of antibodies against GH, SSEA3, and SSEA4, because those antigens are overexpressed on both breast cancer cells and breast cancer stem cells.[19] Also, GH-CRM197 glycoconjugate was shown to elicit antibodies against GH, SSEA3, and SSEA4 in our previous study.[19] Therefore, the antibody response and binding ability to these antigens could be used for evaluating the relative efficacy of azido-GH glycoconjugates as vaccines.[19] Our results showed that **1-CRM197** (N_3 on nonreducing end C6 of Gal on Lac) elicited stronger antibody response against GH antigen than **GH-CRM197** in every sera dilution fold (Figure 2C). Although **3-CRM197** also induced higher antibodies signal to GH antigen than **GH-CRM197**, only 1800× and 16200× of sera dilution showed statistical difference and probably because of the wide variation of antibodies signal from each mouse. **2-CRM197** (N_3 on nonreducing end C6 of Gal on Gb3) and **4-CRM197** (N_3 on nonreducing end C6 of Gal on SSEA3) only induced weak antibodies that cross-reactive to GH antigen. Different glycoconjugates induced antibodies to show similar binding patterns against SSEA3 which share the common SSEA3 epitope with GH (Figure S11A). Interestingly, only **1-CRM197** elicited stronger antibody levels against SSEA4 than **GH-CRM197** (Figure 2D). Since **3-CRM197** only induced robust antibodies against GH and SSEA3 but not SSEA4, GalNAc on GH may be an important epitope for induction of cross-reactive antibodies to SSEA4. It is worth mentioning that **1-CRM197** induced antibodies also cross-react with many sialyl GH series antigens and GH precursors including Gb4 and Gb3 (Figure S4), which are not tumor-specific antigens; therefore, autoimmune issues should be a concern. However, there was no weight loss or other obvious illnesses observed in **1-CRM197** immunized mice. In sum, **1-CRM197** induced significant amounts of antibodies that were cross-reactive to GH, SSEA3, and SSEA4 antigens. The stronger immunogenicity of **1-CRM197** suggested that it could be a better candidate than native GH for vaccine development.

Synthesis of Diazido-GH Glycoconjugates. In our previous immunization study,[35] we found that conjugation of azido-GH with azide at C6 of fucose on GH (**N_3GH**) to carrier protein CRM197 (**N_3GH-CRM197**) can induce a stronger IgG immune response than native **GH-CRM197**. To see if there is synergistic or additive immunogenicity effect for azido-GH analogs with improved immunogenicity, diazido-GH analogs **5** (azide at C6 of fucose and at the nonreducing end C6 of Gal on Lac) and **6** (azide at C6 of fucose and at the nonreducing end C6 of GalNAc on Gb4) were synthesized for evaluation (Figure 1A). The linker of SSEA3 analog **18** was deprotected to afford SSEA3 analog **25** (Figure 3A, Scheme S11). **Gb5** was elongated into **N_3GH**[35] using N_3-fucose as donor with FutC, FKP, PK, and PPA. Using the same method,

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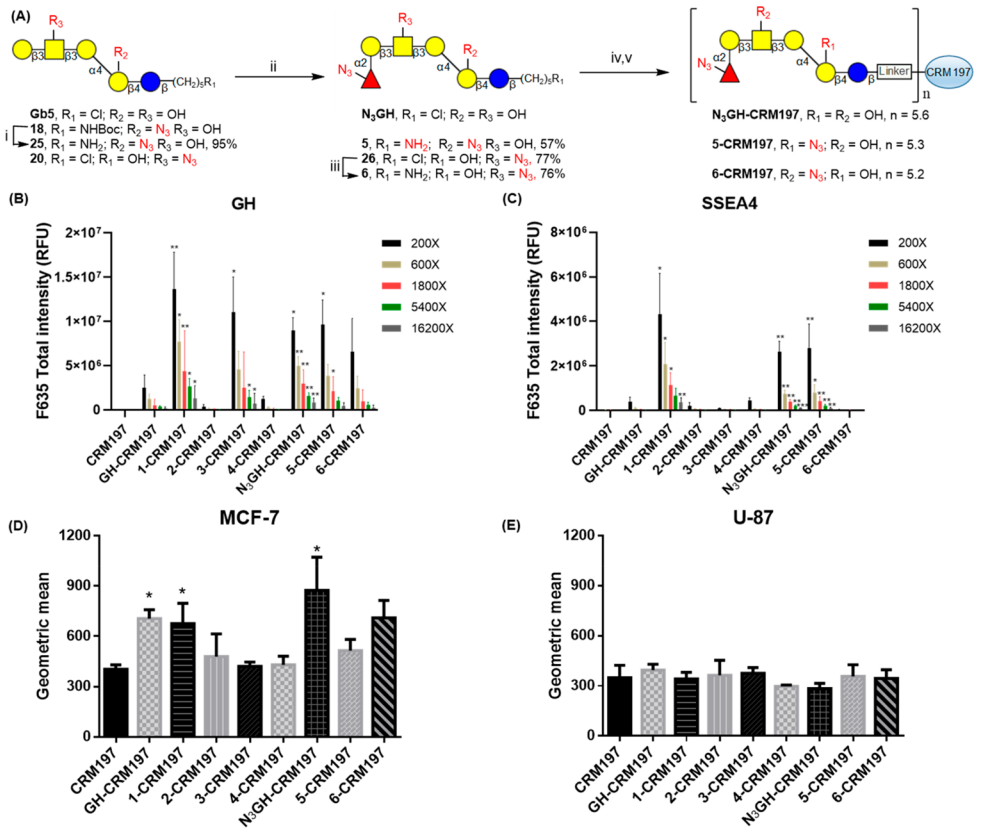

Figure 3. Synthesis of diazido-GH analog vaccines and the binding profile of azido-GH glycoconjugates induced antibodies to the globo-series glycans and GH expressing cells. (A) Synthesis of diazido-GH analog vaccines. (B) IgG level against GH on glycan array. (C) IgG level against SSEA4 on glycan array. Mice number n = 10 in **GH-CRM197**, **1-CRM197**, and **3-CRM197** and n = 5 in **2-CRM197**, **4-CRM197**, **N_3GH-CRM197**, **5-CRM197**, and **6-CRM197**. Each serum was analyzed by glycan array in 5 different dilution fold (200×: black bar; 600×: brown bar; 1800×: red bar; 5400×: green bar; 16200×: gray bar). The secondary antibody used in the array study was Alexa Fluor 647 conjugated goat anti-mouse IgG antibody. Data are means ± SEM (standard error of the mean). Each vaccine was compared with **GH-CRM197** and the comparisons of titers were performed by the Mann−Whitney U test (unpaired). *P < 0.05; **P < 0.01. RFU: relative fluorescence unit. (D) Geometric mean of MCF-7 cells reacting with the antisera from each glycoconjugate immunized mice. (E) Geometric mean of U-87 cells reacting with the antisera from each glycoconjugate immunized mice. For flow cytometry, data from a single experiment representative two experiments was shown with means ± SEM. The comparison between CRM197 and each glycoconjugate was performed by the Mann−Whitney U test (unpaired). *P < 0.05. Reagents and conditions: (i) TFA: H_2O = 9:1; (ii) N_3-fucose, FutC, FKP, PK, PPA, and buffer containing Tris-HCl, $MgCl_2$, PEP, UTP, and ATP; (iii) NH_4OH, reflux; (iv) p-nitrophenyl adipate linker, TEA, and DMF; (v) CRM197 and sodium phosphate buffer (pH 7.9).

azido-SSEA3 analogs **25** and **20** were elongated into diazido-GH analogs **5** and **26** in 57% and 77% yield, respectively (Figure 3A, Scheme S10). The chloride group at the linker of diazido-GH **26** was converted into the amine group to get diazido-GH **6** (Figure 3A, Scheme S12). **N_3-GH** and diazido-GH analogs **5** and **6** were reacted with p-nitrophenyl adipate linker to afford the corresponding half esters **S22**[35]**−S24**, which were conjugated to CRM197 in sodium phosphate buffer (pH 7.9) to obtain **N_3GH-CRM197** and diazido-GH glycoconjugates **5-CRM197** and **6-CRM197** (Figure 3A). The incorporated numbers of GH analogs on CRM197 were monitored by MALDI-TOF and SDS-PAGE (Table S1 and Figure S1) and 5.6, 5.3, and 5.2, respectively.

Glycan Array and Flow Cytometry Analysis of Antisera from Mice Immunized with Azido-GH Glycoconjugates. To study the immunogenicity of diazido- and azido-GH glycoconjugates, **GH-CRM197**, **1-CRM197**, and **3-CRM197** that induced strong antibody responses to GH and the newly synthesized **N_3GH-CRM197**, **5-CRM197**, and **6-CRM197** were used for mice immunization with the same schedule described above (Figure 2B). The sera were collected 10 days after the final shot. These sera and the sera from the first immunization experiment were analyzed by glycan array. We found that all the glycoconjugates elicited strong IgG antibodies to the corresponding antigen (Figures S2−S10) and only low IgM level (Table S3). These indicated that all the glycoconjugates induced a robust T-cell dependent immune response. The antisera induced from **1-CRM197** and **N_3GH-CRM197** have higher IgG response to GH than **GH-CRM197** in every sera dilution fold (Figure 3B). Surprisingly, the more diluted sera (1800×) from these two glycoconjugates (**1-CRM197**, **N_3GH-CRM197**) still showed comparable antibody responses to concentrated sera (200×) from **GH-CRM197**. Notably, **3-CRM197** and **5-CRM197** induced antibodies against GH are only higher than **GH-CRM197** in partial dilution fold, probably due to the wide variation of signal on the glycan array. However, more mice immunized by **3-CRM197** and **5-CRM197** were able to induce antibodies against GH antigen when compared to **GH-CRM197** (**3-CRM197**: 8/10, **5-CRM197**: 5/5, and **GH-CRM197**: 5/10, Table S4). The antibody response induced by diazido-GH glycoconjugates **5-CRM197** and **6-CRM197** against GH were comparable to that by the monoazido-GH glycoconjugates **N_3GH-CRM197**, and no synergistic or additive effect was

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observed. Interestingly, **5-CRM197** induced antibodies that specifically recognized GH, SSEA3, SSEA4, and Gb4 (only low signal, Figure S9) instead of recognizing other sialyl GH series and GH precursors like **1-CRM197** (Figure S4). Due to the elimination of the off-target effect, installation of azide at C′6 of fucose increased the specificity of induced antibodies, preventing the autoimmune issue. Besides, most glycoconjugates except **3-CRM197** and **6-CRM197** induced antibodies exhibited similar pattern in recognizing SSEA3 (Figure S11A and B) and SSEA4 (Figure 3C). As in our earlier results (Figure 2C), **3-CRM197** and **6-CRM197** that had azido modification on the nonreducing end GalNAc of Gb4 did not induce cross-reactive antibodies to SSEA4 (Figure 3C). This result again indicated that GalNAc on GH may be a crucial epitope for induction of antibodies cross-reactivity toward SSEA4.

Since some azido-GH glycoconjugates induced excellent IgG antibody binding to GH and SSEA3, the IgG subtypes were analyzed by glycan array and found that the predominant subtype in various azido-GH glycoconjugates induced antisera was IgG1 (Figure S11C), which is mainly induced by Th2-type responses.[54,55] Overall, the results showed that azido-GH analogs containing the azido group at the nonreducing end C6 of the Gal on Lac (**1**), at C6 of the terminal fucose (**N_3GH**), and at both these two sites (**5**) elicited stronger immunogenicity than the native GH did. The antibodies induced by these three glycoconjugates not only bind to their self-antigens but also cross-react with GH, SSEA3, and SSEA4 antigens on the glycan array.

To see if the antisera induced by azido-GH glycoconjugates can bind to the GH antigen on the surface of cancer cells, we reacted all the antisera from each glycoconjugate with GH-expressing cancer cells MCF-7 and examined the reaction by flow cytometry. The antisera from mice immunized with glycoconjugates **GH-CRM197**, **1-CRM197**, and **N_3GH-CRM197** showed binding to MCF-7 cells (Figures 3D and S12A). In contrast, all the antisera elicited from each glycoconjugate did not recognize the GH-negative U-87 cells (Figures 3E and S12B). It should be mentioned that the control mice were immunized with CRM197 only but not with adjuvant, which might influence the sera background reactivity on cancer cells in the experimental mice and affect the flow cytometry results. Since the flow cytometry signals were only moderate, we further used competition assay to validate the binding between MCF-7 and antisera induced by **GH-CRM197**, **1-CRM197**, **N_3GH-CRM197**, and **6-CRM197** were specific to Globo H on cancer cells. Adding GH or the corresponding GH analog to the antisera-MCF-7 mixture would significantly inhibit the recognition of antisera to MCF-7 (Figure S13A−D). In contrast, those glycoconjugate-induced antisera that did not show obvious binding to MCF-7 did not show significant changes in the GH competition assay (Figure S13E). The binding patterns in glycan array and flow cytometry were not completely proportional, possibly due to the linker effect, which was recognized as a critical issue for antibody diversity and tumor cell binding.[56]

In sum, these results indicated that the azido-GH glycoconjugates were able to induce antibodies that recognized GH on GH-positive cancer cells.

CONCLUSION

In this study, we replaced specific hydroxyl groups with azido group to prepare various azido-GH analogs for vaccine conjugation and their immunogenicity evaluation.

Specifically, an efficient chemoenzymatic strategy for the synthesis of azido-GH analogs was developed. Using galactosidase to oxidize the nonreducing end C6-OH of Gal and GalNAc on GH precursors, the corresponding aldehyde was transformed to the azido group as substrates for glycosyltransferase- and SNR-catalyzed glycosylation. These azido-GH analogs were conjugated to CRM197 as vaccine candidates for immunization in mice with C34 adjuvant designed to enhance immune response and class switch. Glycan array analysis of the antisera from mice immunized with azido-GH conjugates showed that **1-CRM197** and **5-CRM197** elicited stronger IgG response that recognized GH, SSEA3, and SSEA4 antigens than native **GH-CRM197** did. The diazido-GH analogs **5-CRM197** and **6-CRM197** induced comparable antibody titers to **N_3GH-CRM197**, but no synergistic effect was observed. Furthermore, the glycoconjugates **1-CRM197** and **N_3GH-CRM197** induced antibodies that recognize GH expressing cancer cells. Among these vaccine candidates, **1-CRM197** is the most effective.

In summary, through a systematic study of various azido-GH analogs prepared by an efficient chemoenzymatic method, we have identified **1-CRM197** glycoconjugate as better cancer vaccine candidates than GH conjugate for active immunization. Work is in progress to further evaluate this vaccine candidate in animal models for a possible translation to human trials.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.1c01277.

General method, supplementary schemes, synthetic protocols, and glycan array analysis (PDF)

Analytical data for new compounds (PDF)

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