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Linear synthesis and immunological properties of a fully synthetic vaccine candidate containing a sialylated MUC1 glycopeptide†

Pamela Thompson,[ab] Vani Lakshminarayanan,[c] Nitin T. Supekar,[ab] Judy M. Bradley,[d] Peter A. Cohen,[d] Margreet A. Wolfert,[a] Sandra J. Gendler*[c] and Geert-Jan Boons*[ab]

A strategy for the linear synthesis of a sialylated glycolipopeptide cancer vaccine candidate has been developed using a strategically designed sialyl-Tn building block and microwave-assisted solid-phase peptide synthesis. The glycolipopeptide elicited potent humoral and cellular immune responses. T-cells primed by such a vaccine candidate could be restimulated by tumor-associated MUC1.

Mucins are high molecular weight glycoproteins containing numerous *O*-linked glycans that are found on the apical surface of epithelial cells.[1] They play key roles in the protection, repair and survival of the epithelia and suppress inflammatory responses at the interface with the environment.[1,2] Deregulation of the biosynthesis of mucins has been linked to epithelial cancers, including those of breast, ovary, lung and pancreas, which commonly overexpress mucins to exploit their role in promoting cell growth and survival.

The mucin MUC1 is one of the most promising targets for the development of immuno-therapies for cancer.[3] It is a heavily glycosylated type 1 transmembrane mucin that is composed of a cytoplasmic signaling peptide, a transmembrane domain and an ectodomain composed of a variable number tandem repeats of twenty amino acids (TAPPHAGVTSAPDTRPAPG). Each tandem repeat has five potential sites for *O*-glycosylation,[4] and the pattern of glycosylation depends on the type and physiological state of the tissue.[5] Tumor-associated MUC1 is aberrantly glycosylated due to a lack of core 1,3-galactosyl transferase (T-synthase),[6] producing truncated carbohydrate structures such as Tn (αGalNAc-Thr) and STn (αNeu5Ac-(2,6)-αGalNAc-Thr).

[a] *Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Road, Athens, GA 30602, USA. E-mail: gjboons@ccrc.uga.edu; Fax: +1 706-542-4412*
[b] *Department of Chemistry, The University of Georgia, Athens, GA 30602, USA*
[c] *Department of Biochemistry and Molecular Biology and Immunology, Mayo Clinic College of Medicine and Mayo Clinic Comprehensive Cancer Center, Scottsdale, AZ 85259, USA. E-mail: gendler.sandra@mayo.edu*
[d] *Department of Hematology and Oncology, Mayo Clinic College of Medicine and Mayo Clinic Comprehensive Cancer Center, Scottsdale, AZ 85259, USA*

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Humoral and cellular immune responses against tumor-associated MUC1 have been observed in cancer patients. The presence of circulating antibodies against MUC1 at the time of cancer diagnosis has been correlated with a favorable disease outcome in breast cancer patients.[7] Furthermore, cytotoxic T-lymphocytes (CTLs) isolated from patients with breast carcinoma can recognize epitopes present on MUC1 tandem repeat peptide.[8] The inherent immunological properties of tumor-associated MUC1 have stimulated the development of cancer immune therapies;[4] however, it has been difficult to design therapeutic vaccines that can elicit relevant IgG antibodies and CTLs against tumor-associated MUC1.

We have shown that a three-component cancer vaccine composed of a tumor-associated carbohydrate B-cell epitope, a promiscuous T_{helper} peptide epitope, and a Toll-like receptor (TLR) agonist circumvents immune suppression caused by a carrier protein.[9] The exceptional immunogenic properties of this vaccine were attributed to the absence of unnecessary features that may be antigenic and cause immune suppression, yet the vaccine contains all the relevant epitopes required for eliciting relevant innate, cellular and humoral immune responses. It was found that attachment of the TLR2 agonist Pam_3CysSK_4[10] to the B- and T-cell epitopes was essential for optimal activity. Furthermore, a similar vaccine having the Pam_3CysSK_4 moiety replaced by CpG, which is a TLR9 agonist, elicited inferior responses.[11] We have also applied the technology toward the synthesis of immunogens to generate monoclonal antibodies specific for *O*-GlcNAc.[12]

We have prepared glycolipopeptide vaccine candidates by a combination of solid-phase peptide synthesis (SPPS) and liposome-mediated native chemical ligation (NCL).[13] Several other groups have emulated the multicomponent vaccine technology using (glyco)peptides covalently attached to Pam_3CysSK_4 or other inbuilt adjuvants.[14] In these approaches, the vaccines were prepared by various ligation strategies in combination with unnatural linkers to attach (glyco)peptides to a TLR agonist. For example,[14e] Cu-catalyzed alkyne–azide cycloaddition has been used to attach MUC1-derived glycopeptides modified at the N-terminus with an azido moiety to Pam_3CysK_4 containing an oligo-ethylene glycol spacer extended

by a terminal alkyne. Although these approaches offer convenient entries into fully synthetic vaccine candidates, the linker may induce antigenic responses[15] thereby causing immunosuppression of the tumor-associated glycopeptide. It may also interfere with antigenic processing of the peptide.

Although the liposome-mediated NCL is attractive for the preparation of three-component vaccine candidates having simple saccharides, it failed to provide the required product when glycopeptides were employed having more complicated saccharide moieties such as STn. The ability to incorporate different types of glycan in cancer vaccines will make it possible to personalize treatment. In this respect, 25–30% of breast cancers are STn positive, which correlates with poor prognosis.[16] Furthermore, a block synthetic approach using liposome-mediated NCL is less suitable for large scale synthesis of glycolipopeptides required for future clinical studies. Thus, there is an urgent need to develop a linear synthetic strategy that avoids unnatural linkers and can give glycolipopeptide vaccines modified by a complex carbohydrate such as STn.

We report here an efficient synthesis of glycolipopeptide **1** that is composed of a MUC1 glycopeptide containing the sialyl Tn moiety, a helper T-cell epitope derived from the polio virus[17] and the TLR2 ligand Pam_3CysSK_4 (Fig. 1). It employs a strategically designed sialyl-Tn building block that allowed for the rapid linear assembly of the target glycolipopeptide using microwave-assisted solid-phase peptide synthesis. In parallel, compound **2** was prepared having a Tn moiety and compound **3**, which is a control to account for adjuvant effects. We demonstrate here that a three-component vaccine **1** having the STn moiety can elicit potent humoral and cellular immune responses in a mouse transgenic for human MUC1.

Our first attempt to synthesize a MUC1-derived glycopeptide having an STn moiety involved the use of a threonine derivative having a sialosyl residue protected as a methyl ester.[18] Although great care was taken in the deprotection of the methyl ester of the sialoside using mild basic conditions, it resulted mainly in β-elimination of the glycan. A benzyl ester protected sialic acid derivative[19] was not considered because its deprotection would be difficult to accomplish on the target glycolipopeptides. Therefore, sialyl-Tn derivative **15** was designed which is compatible with Fmoc-based MW-SPPS and has the carboxylic acid of sialic acid protected as an allyl ester which can be removed under mild conditions that was expected to avoid β-elimination.

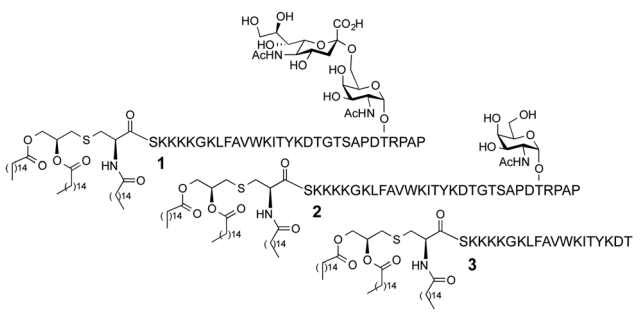
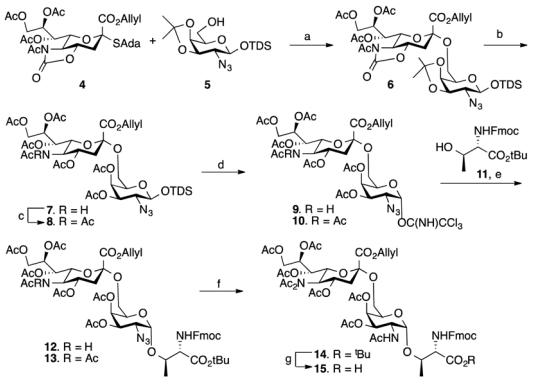


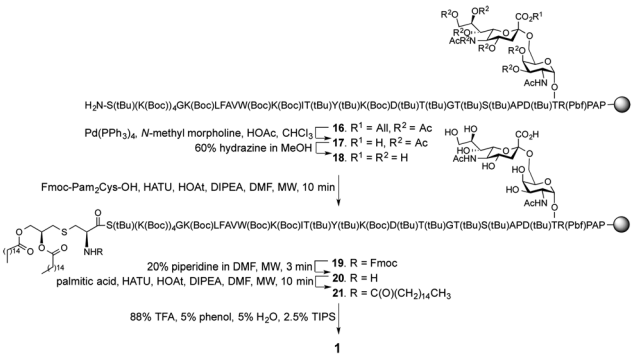
Fig. 1 Multicomponent vaccine candidates having an STn (**1**) or Tn (**2**) moiety. Compound **3** is a control to account for adjuvant effects.



Scheme 1 Reagents and conditions. (a) NIS, TfOH, DCM/MeCN, −78 °C (86%); (b) NaOMe, AllylOH; then 70% AcOH (aq), 70 °C; then Ac_2O, Py (65% over 3 steps); (c) isopropenyl acetate, CSA, 65 °C (99%); (d) HF–pyridine, THF; then CCl_3CN, DBU, (76% over 2 steps); (e) TMSOTf, Et_2O, 0 °C (85%); (f) Zn, $CuSO_4$, THF, Ac_2O, AcOH (65%); (g) TFA/DCM (1/1, v/v) (99%).

Previously, we have found that modification of the C-5 acetamido moiety of sialic acid by *N*-acetylacetamido or *N*-trifluoroacetyl greatly improves glycosyl donor and acceptor properties and provide glycosides with improved α-anomeric selectivities.[20] Several alternative strategies for C-5 modification have been reported, and in particular, 1-adamantylthio sialosides that contain an *N*-acetyl-5-*N*-4-*O*-oxazolidinone give excellent yields and α-selectivities in glycosylations of various glycosyl acceptors using NIS–TfOH as the activator.[21] As expected, glycosylation of donor **4** with galactosyl acceptor **5** using NIS/TfOH as the activator at −78 °C in a mixture of DCM/MeCN provided disaccharide **6** in an excellent yield as only the α-anomer (Scheme 1). The oxazolidinone and isopropylidene protecting groups of **6** were removed by subsequent treatment with sodium methoxide in allyl alcohol followed by aqueous acetic acid at 70 °C, and the amino group and alcohols of the resulting compound were acetylated using acetic anhydride in pyridine to provide sialyl disaccharide **7**. The thexyl dimethylsilyl (TDS) protecting group of **7** was removed with HF–pyridine and the resulting lactol was converted into a trichloroacetimidate **9** by treatment with trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Glycosylation of **9** with threonine **11**[22] resulted in an inseparable mixture of α–β anomers. Surprisingly, we found that installing an *N*,*N*-diacetyl moiety the C-5 amino group of on sialic acid[20a] to give donor **10** resulted in a stereoselective glycosylation with **11** to provide **13** in high yield. Reduction of the azide of **13** using Zn/Cu couple in the presence of acetic anhydride, followed by removal of the *tert*-butyl (^{t}Bu) protecting group of the threonine moiety of the resulting compound **14**, gave the desired properly protected STn derivative **15**.

We envisaged a synthetic strategy for **1** in which the full length glycopeptide is first assembled by SPPS followed by subsequent removal of the allyl ester and acetyl protecting groups, and then installation of the Pam_3CysK_4 moiety, and finally concomitant side chain deprotection and release of the compound from the resin. Such a strategy will avoid cleavage of palmitoyl esters of Pam_3CysK_4 during the deacetylation step while the acetyl protecting groups of the hydroxyls and the acetamido functionality of sialic acid can be removed on resin without affecting the allyl ester.



Scheme 2 Microwave-assisted SPPS of glycolipopeptide **1**.

Previously, we had observed that a linear solid phase peptide synthesis protocol for the preparation of compounds such as **2** gave products that were difficult to purify to homogeneity.[9d] It is known that microwave-assisted synthesis of (glyco)peptides reduces reaction times while providing (glyco)peptides of high purity.[23] Therefore, we were compelled to investigate whether this technology would allow for the linear synthesis of vaccine candidate **1** and **2** (Scheme 2). Using Rink Amide AM LL resin, the first four amino acids were introduced using a CEM Liberty 12-channel automated microwave peptide synthesizer, which utilizes an 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) activation protocol. Glycosylated amino acid **15** was introduced manually using 1-[dis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) under microwave irradiation. The resin was then returned to the synthesizer for further peptide elongation to give **16**. The resin was removed from the synthesizer and treated with $Pd(PPh_3)_4$, in $CHCl_3$, acetic acid, and *N*-methyl morpholine to remove the allyl ester of sialic acid (→**17**).[24] Next, 60% hydrazine in methanol was added to remove the acetyl moieties[9d] of the disaccharide to give resin bound glycopeptide **18**. The Fmoc-Pam_2Cys and palmitic acid were coupled manually using HATU/HOAt in the presence of *N*,*N*-diisopropylethylamine (DIPEA) in DMF under microwave irradiation to give **21**. Finally, amino acid side chain deprotection and cleavage from the resin was accomplished using 88% TFA, 5% phenol, 5% H_2O and 2% TIPS. The glycolipopeptide **1** was obtained following purification by RP-HPLC using a C4 column. In a similar manner, compound **2** was prepared employing N^{α}-Fmoc-Thr(AcO_3-α-D-GalNAc) as a building block. Prior to the coupling of FmocPam$_2$Cys, a small amount of glycopeptide was released from the resin and analysed by MS, which showed the absence of incomplete sequences.

Next, attention was focused on exploring the immunological properties of the vaccines. Groups of MUC1.Tg mice (C57BL/6; H-2^b) that express human MUC1[25] were immunized with liposomal preparation of **1**, **2** and **3** and empty liposomes five times intradermally at the base of the tail at biweekly intervals. One week after the last immunization, the mice were sacrificed and the humoral immune responses were assessed by titers of MUC1-specific antibodies and the ability of the antisera to lyse MUC1-bearing tumor cells. In addition, cellular immune responses were evaluated by ELISPOT assay.

Table 1 ELISA anti-MUC1 antibody titers in endpoint serum samples

	IgG total	IgG1	IgG2a	IgG2b	IgG3	IgM
1	12 700	3700	900	2800	5100	200
2	29 200	8800	3500	13 200	5600	100
3	800	100	0	0	0	100
EL	1400	0	0	400	0	0

Antibody titers are presented as median values for groups of mice. ELISA plates were coated with BSA-MI-CTSAPDT(Neu5Ac-α2,6-αGalNAc)RPAP conjugate for anti-MUC1(STn) antibody titers for **1**, **3** and empty liposomes (EL) or BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate for anti-MUC1(Tn) antibody titers for **2**.

Compounds **1** and **2** elicited robust IgG antibody titers and subtyping indicated a mixed Th1/Th2 response (Table 1 and Fig. S1 in the ESI†). Antibody-dependent cell-mediated cytotoxity (ADCC) was examined by labeling MUC1-expressing mammary cancer cells with ^{51}Cr, followed by the addition of antisera and cytotoxic effector cells (NK cells) and measurement of released ^{51}Cr. The antisera obtained by immunization with **1** and **2** significantly increase cancer cell lysis compared to control (Fig. 2A).

To assess the ability of the vaccine candidates to activate CTLs, $CD8^+$ T-cells from spleens were isolated by magnetic cell sorting and incubated with irradiated dendritic cells (DCs) pulsed with the immunizing glycopeptides on ELISPOT plates. As expected, vaccine candidates **1** and **2** exhibited robust T-cell responses compared to controls, and, surprisingly, the response for STn-containing derivative **1** was somewhat stronger compared to that of the Tn-containing compound **2** (Fig. 2B). To further evaluate T-cell responses, lymph node derived T-cells expressing low levels of CD62L were cultured for 7 days in the presence of DCs pulsed with the corresponding immunizing construct and the resulting cells were analyzed by intracellular cytokine staining (ICC) for the presence of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T-cells. It was found that both $CD4^+$ and $CD8^+$ T-cells of mice immunized with liposomes containing **1** and **2** were activated by the MUC1 epitope (Fig. S2 of the ESI†). Compound **3** was, however, a poor activator of $CD4^+$ T-cells indicating that residues of the MUC1 epitope contribute to the helper T-epitope. Furthermore, when $CD62L^{low}$ T-cells of

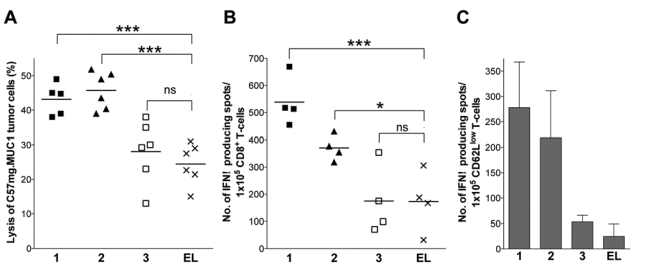


Fig. 2 (A) Induction of antibody-dependent cell-mediated cytotoxicity (ADCC) with C57mg MUC1 tumor cells. (B) Induction of cytotoxic $CD8^+$ T-cell response by analyzing MUC1-specific IFNγ spot formation without *in vitro* stimulation. Each data point in (A) and (B) represents an individual mouse and the horizontal lines indicate the mean for the group of mice. Asterisks indicate statistically significant difference (* $P < 0.05$, *** $P < 0.001$) and ns no significant difference. (C) Reactivation of cytotoxic $CD62L^{low}$ T-cell response to tumor-associated MUC1 by analyzing MUC1-specific IFNγ spot formation after *in vitro* stimulation with B16.MUC1 cells (mean ± SEM).

the immunized mice were incubated with dendritic cells (DCs) pulsed with the immunizing peptides and then exposed to melanoma B16.MUC1 cells on ELISPOT plates, vaccine candidates **1** and **2** exhibited a robust response compared to control (Fig. 2C), highlighting that T-cells primed by the vaccine can be reactivated and expanded by tumor-associated MUC1.

In summary, we report here the first linear preparation of a fully synthetic three-component cancer vaccine candidate devoid of any artificial linkers. Key strategic issues included the removal of the acetyl esters of the glycan moiety prior to the installation of the $Pam_2CysFmoc$ moiety, the use of a strategically chosen STn building block that has the carboxylic acid of the sialoside protected as an allyl ester which could be removed under neutral conditions without causing β-elimination of the *O*-glycan, and microwave-assisted solid-phase peptide. Although it has been suggested that STn can suppress immune responses,[26] we have found that a fully synthetic three-component vaccine containing this epitope can elicit potent humoral as well cellular immune responses. Furthermore, it is shown that T-cells primed by such a vaccine can be restimulated by tumor-associated MUC1, which is highly significant because such restimulation of T-cells will lead to their expansion at the site of the tumor and be more able to eliminate cancer cells. Previously, an STn–KLH conjugate was developed as a vaccine for metastatic breast cancer,[27] which failed in phase III clinical trails.[28] The poor immunogenicity of this vaccine is likely due to immunosuppression caused by the carrier protein. Other STn-containing vaccine candidates have been examined in wild type mice, which do not establish breaking of tolerance.[14i,19] We have shown that a carefully designed vaccine candidate containing STn can break immune tolerance and induce humoral and cellular immune responses.

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