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Synthesis and Immunological Evaluation of a Multicomponent Cancer Vaccine Candidate Containing a Long MUC1 Glycopeptide

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A fully synthetic MUC1-based cancer vaccine was designed and chemically synthesized containing an endogenous helper T-epitope (MHC class II epitope). The vaccine elicited robust IgG titers that could neutralize cancer cells by antibody-dependent cell-mediated cytotoxicity (ADCC). It also activated cytotoxic T-lymphocytes. Collectively, the immunological data demonstrate engagement of helper T-cells in immune activation. A synthetic methodology was developed for a penta-glycosylated MUC1 glycopeptide, and antisera of mice immunized by the new vaccine recognized such a structure. Previously reported fully synthetic MUC1-based cancer vaccines that elicited potent immune responses employed exogenous helper T-epitopes derived from microbes. It is the expectation that the use of the newly identified endogenous helper T-epitope will be more attractive, because it will activate cognate $CD4^+$ T-cells that will provide critical tumor-specific help intratumorally during the effector stage of tumor rejection and will aid in the generation of sustained immunological memory.

During malignancy, biosynthesis of the mucin MUC1 is upregulated by as much as 50-fold, resulting in a loss of polarization.[1] Furthermore, in tumor-associated MUC1, the variable number of tandem repeats (VNTR) of the extracellular domain, which harbor five potential sites of O-glycosylation, are underglycosylated, revealing peptide epitopes that are shielded by complex glycans in healthy tissue.[2] The overexpression and aberrant glycosylation of MUC1 correlates with invasive cell growth and metastasis.[3] It disrupts cell–cell adhesion, contributes to escape of tumor immune surveillance, and provides chemoresistance and anti-apoptotic properties. MUC1 is also an oncoprotein and can activate cell signaling events that contribute to cancer progression. Humoral, as well as cellular, immunity against tumor-associated MUC1 have been observed in cancer patients and correlate with favorable disease outcomes. These observations have created considerable interest in the development of immune-therapies that target tumor associated MUC1.[4]

Previously, we reported a fully synthetic multicomponent vaccine composed of an aberrantly glycosylated MUC1 peptide, a peptide helper T-epitope derived from poliovirus, and the immunoadjuvant Pam_3CysSK_4 (**1**, Scheme 1), which can disrupt immune tolerance in human MUC1 transgenic mice.[5] The candidate vaccine elicited robust IgG antibody responses that could neutralize cancer cells by antibody-dependent cell-mediated cytotoxicity (ADCC). It also activated cytotoxic T-lymphocytes (CTLs) and was efficacious in a mouse model for mammary cancer. Structure–activity relationship (SAR) studies showed that optimal immune responses were achieved when

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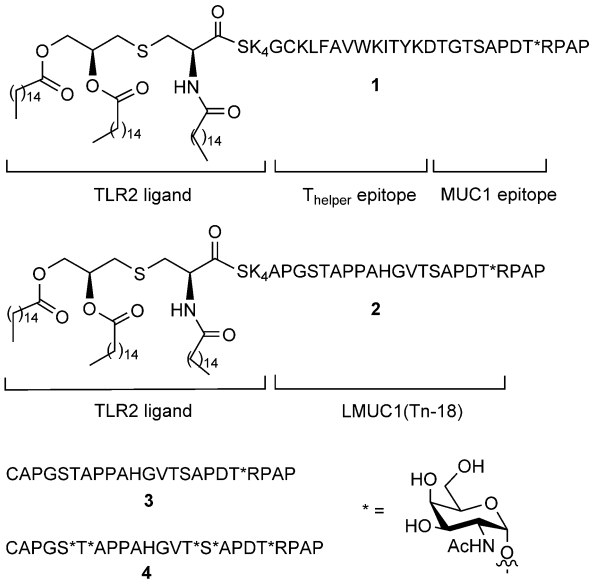


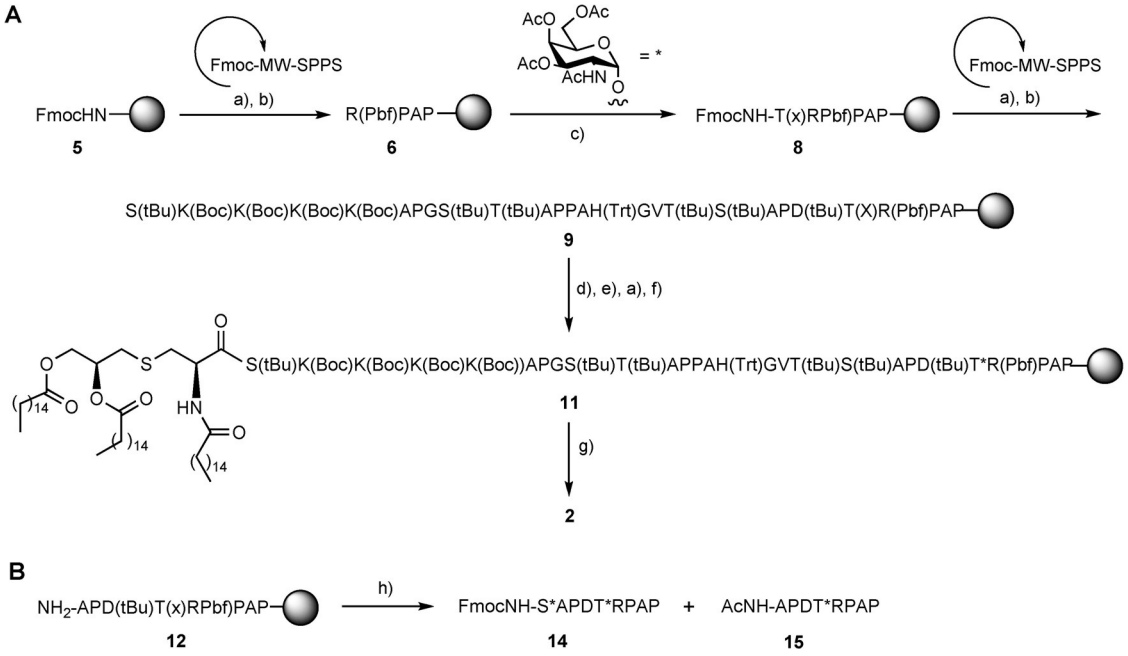
Scheme 1. Glycopeptides and glycolipopeptides for immunization studies.

the three components were covalently linked and the B-epitope was presented at the C terminus of the glycolipopeptide. Furthermore, we found that an immunogen with the Pam_3CysSK_4 moiety replaced by CpG, which is a TLR9 agonist, elicited inferior immune responses.[6] A fully synthetic multicomponent vaccine containing STn also elicited potent humoral and cellular immune responses.[7] Several other multicomponent MUC1-based cancer vaccines that exhibit some of the properties of compound **1** have been described.[8]

We envisaged that the long MUC1-derived glycopeptide, APGSTAPPAHGVTSAPDT(*O*-GalNAc)RPAP, having a number of unique properties, would offer a tantalizing antigen for cancer vaccine development,. It was anticipated to contain multiple epitopes that can activate B-cells, cytotoxic T-lymphocytes, and helper T-cells. The glycopeptide SAPDT(*O*-GalNAc)RPAP is a well-established B-epitope that can elicit relevant humoral responses.[9] It is also an MHC class I (Kb) epitope that can activate cytotoxic T-lymphocytes.[10] Glycosylation of this peptide is important for eliciting antibodies and activating $CD8^+$ cells that can neutralize MUC1-expressing cancer cells.[5b] In addition, the peptides STAPPAHGV and PAHGVTSA are predicted MHC class I (Ab) epitopes.[10] We analyzed the full-length tandem repeat of MUC1 by Rankpep,[11] which indicated that the peptide STAPPAHGVTSA might function as a promiscuous helper T-epitope (class II). This was supported by the finding that MUC1-specific $CD4^+$ T cells could be elicited by employing a MUC1-derived peptide of 100 amino acids or by tumor challenge.[12] Although MHC class II epitopes derived from tumor antigens may not activate T-cell as effectively as exogenous epitopes such as the one derived from poliovirus used in vaccine **1**,[5] they will induce cognate $CD4^+$ T-cells that will provide important help during secondary immune responses. A number of studies have confirmed the attractiveness of using self-epitopes for stimulation of $CD4^+$ T-cell responses.[13]

Vaccine candidate **2** was prepared to examine whether the MUC1-derived glycopeptide (APGSTAPPAHGVTSAPDT(*O*-GalNAc)RPAP) could activate $CD4^+$ and $CD8^+$ T-cells and induce IgG antibodies. The threonine moiety of the SAPDTRPAP epitope was modified by GalNAc, because previous studies have shown that glycosylation of this residue is important for eliciting tumor-relevant CTLs and antibodies.[5b,14] The other serine and threonine moieties were not modified, because glycosylation can block proteolytic processing of the tandem repeat, which might compromise presentation of MHC1 and MHCII epitopes required for activation of $CD4^+$ and $CD8^+$ T-cells, respectively.[15] In addition, the monoglycosylated glycopeptide **3** was prepared, which has an N-terminal cysteine moiety for conjugation to a carrier protein to evaluate the presence of serum antibodies by ELISA. A similar pentaglycosylated tandem repeat (**4**) was prepared to examine whether antibodies elicited by **2** could recognize highly glycosylated MUC1. In this respect, tumor-associated MUC1 is characterized by high-density glycosylation of the tandem repeat, and it is known that glycosylation of MUC1 epitopes can influence antibody recognition.[16]

Synthesis of the monoglycosylated vaccine candidate **2** was straightforward and entailed coupling of the first four amino acids on a Rink amide AM LL resin (**5**) by using a CEM Liberty 12-channel automated microwave peptide synthesizer and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the activation protocol (Scheme 2 A). The glycosylated amino acid *N*-Fmoc-Thr(AcO_3-α-D-GalNAc (**7**) was introduced manually under microwave heating with 1-[dis(dimethyl amino)methylene]-1*H*-



Scheme 2. A) Chemical synthesis of glycolipopeptide **2** and B) chain termination during synthesis of glycopeptide **4**. a) 20% 4-methylpiperidine, DMF, MW, 3 min; b) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW, 5 min; c) FmocNH-T*-COOH (**7**; 2 equiv), HATU, HOAt, DIPEA, DMF, MW, 10 min; d) 70% hydrazine in MeOH; e) Fmoc-Pam_2Cys-OH (**10**), HATU, HOAt, DIPEA, DMF, MW, 10 min; f) palmitic acid, HATU, HOAt, DIPEA, DMF, MW, 10 min; g) TFA/phenol/H_2O/TIPS (88:5:5:2); h) FmocNH-S*-COOH (**13**; 2 equiv), HATU, HOAt, DIPEA, DMF, MW, 10 min.

1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) as the activation reagent. The resulting resin, **8**, was elongated in the synthesizer under standard coupling conditions to give resin-bound glycopeptide **9**, which was treated with 60% hydrazine in methanol to remove the acetyl esters[5a] of GalNAc. Manually, Fmoc-Pam_2Cys (**10**) was coupled to the N terminus of **9** by using HATU/HOAt in the presence of *N*,*N*-diisopropylethylamine (DIPEA) in DMF under microwave heating, which was followed by removal of the Fmoc protecting group and further acylation of the resulting amine with palmitic acid to give **11**. The resin-bound glycolipopeptide was released from the resin by treatment with a mixture of TFA, phenol, H_2O, and TIPS (88:5:5:2, *v*/*v*/*v*/*v*), with simultaneous deprotection of the amino acid side chain protecting groups. Purification by HPLC on a reversed-phase C_4 column gave homogeneous **2**. A small amount of glycopeptide **9** was released from the resin prior to further coupling, and analysis by MS showed the absence of incomplete sequences. Glycopeptide **3** was prepared according to a similar coupling strategy (Scheme S1 in the Supporting Information).

Preparation of pentaglycosylated glycopeptide **4** was more challenging. Installation of the second glycosylated amino acid resulted in product **14** and by-product **15** (10–20%), in which the N-terminal amino group of the starting peptide was acetylated (Scheme 2B). Similar side reactions were observed during the coupling of the third, fourth, and fifth glycosylated amino acids, resulting in a very low yield of desired product. It is probable that the high reaction temperature employed in the microwave-assisted coupling step and the longer reaction time caused acetyl migration from the glycosylated amino acids to the N-terminal amine of the starting peptide. This problem could be addressed by reducing the amount of glycosylated amino acid (1.1 equiv), lowering the reaction temperature from 75 to 60 °C, and shortening the reaction time (10 to 5 min) during the coupling steps of the glycosylated amino acids (Scheme S2). A Kaiser test was performed after coupling of each glycosylated amino acid, and unreacted amino groups were capped by using acetic anhydride. After assembly of the full tandem repeat, an N-terminal cysteine residue was introduced, and the resulting compound was deprotected and purified according to standard procedures to give compound **4** in good overall yield.

Glycopeptides **3** and **4**, containing an N-terminal cysteine residue, were conjugated to BSA-modified maleimides (BSA-MI) according to a standard protocol. The conjugates were purified by spin filtration, the coupling efficiency was determined by MALDI-TOF mass spectrometry (~ten copies of glycopeptide/BSA), and protein concentration was determined by Bradford protein assay.

Humoral and cellular immune responses elicited by vaccine candidate **2** were explored in MUC1.Tg mice (C57BL/6; H-2^b) expressing human MUC1.[17] A group of five mice were intradermally immunized with liposomal preparations of compound **2** four times at biweekly intervals. Pam_3CysSK_4 and empty liposomes were administered as negative controls. One week after the last immunization, the mice were sacrificed, and humoral and cellular immune responses were evaluated by examining titers of MUC1-specific antibodies, the ability of the antisera to lyse MUC1-bearing tumor cells, and ELISPOT assay with T-cells.

Mice immunized with compound **2** elicited robust IgG antibody titers compared to Pam_3CysSK_4 and empty liposomes. The elicited antibodies recognized both mono- and pentaglycosylated full-length MUC1 epitopes **3** and **4**, respectively (Table 1). IgG subtyping showed the presence of IgG1, IgG2a, and IgG2b antibodies, indicating a mixed Th1/Th2 response (IgG2a/b represents a Th1 response, and IgG1 represents a Th2 response). Very low titers of IgM antibodies were observed, demonstrating efficient class switching from low-affinity IgM to high-affinity IgG antibodies. These results indicated that the vaccine candidate embedded an MHCII epitope that could activate helper T-cells.

Only antisera obtained by immunization with **2** showed significant binding to C57mg mammary cancer cells transfected with MUC1 (Figure S2). Wild-type C57mg cells, known to lack MUC1 expression, did not show binding with antisera **2** (Figures S1 and S2). Antibody-dependent cell-mediated cytotoxicity (ADCC) was examined by labeling MUC1-expressing C57mg 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 **2** significantly increased cancer cell lysis compared to the control groups immunized with empty liposomes or Pam_3CysSK_4 (Figure 1A).

To assess the ability of **2** to activate CTLs, $CD62L^{low}$ T-cells were isolated by magnetic cell sorting and incubated with dendritic cells (DCs) without in vitro stimulation and then analyzed for MUC1-specific IFNγ spot formation on ELISPOT plates. It was observed that vaccine candidate **2** had robustly activated T-cells compared to the control groups (Figure 1B). The T-cell response was further evaluated for MUC1 epitope recognition of $CD4^+$ and $CD8^+$ T-cells by culturing T-cells derived from lymph nodes and expressing low levels of CD62L for seven days in the presence of DCs pulsed with the corresponding immunizing construct. The resulting cells were analyzed by intracellular cytokine (ICC) staining for the presence of $CD4^+$ $IFN\gamma^+$ (Figure 1C) and $CD8^+IFN\gamma^+$ T-cells (Figure 1D). As antici-

Table 1. ELISA anti-LMUC1 antibody titers in endpoint serum samples.

	IgG total	IgG1	IgG2a	IgG2b	IgG3	IgM
BSA-MI-**3**[a]						
2	23200	29200	1200	3600	5800	300
Pam_3CysSK_4	300	0	0	0	0	0
EL	2000	600	300	800	500	0
BSA-MI-**4**[b]						
2	21700	29900	2000	4800	3500	500
Pam_3CysSK_4	0	0	0	0	0	100
EL	1200	500	600	400	300	0

Antibody titers are presented as median values for groups of mice. ELISA plates were coated with [a] BSA-MI-CAPGSTAPPAHGVTSAPDT (αGalNAc)R-PAP (BSA-MI-**3**) conjugate for anti-LMUC1(Tn18) antibody titers or [b] BSA-MICAPGS(αGalNAc)T(αGalNAc)APPAHGVT(αGalNAc)S(αGalNAc)APDT-(αGalNAc)RPAP (BSA-MI-**4**) conjugate for anti-LMUC1(5Tn) antibody titers. EL: empty liposomes.

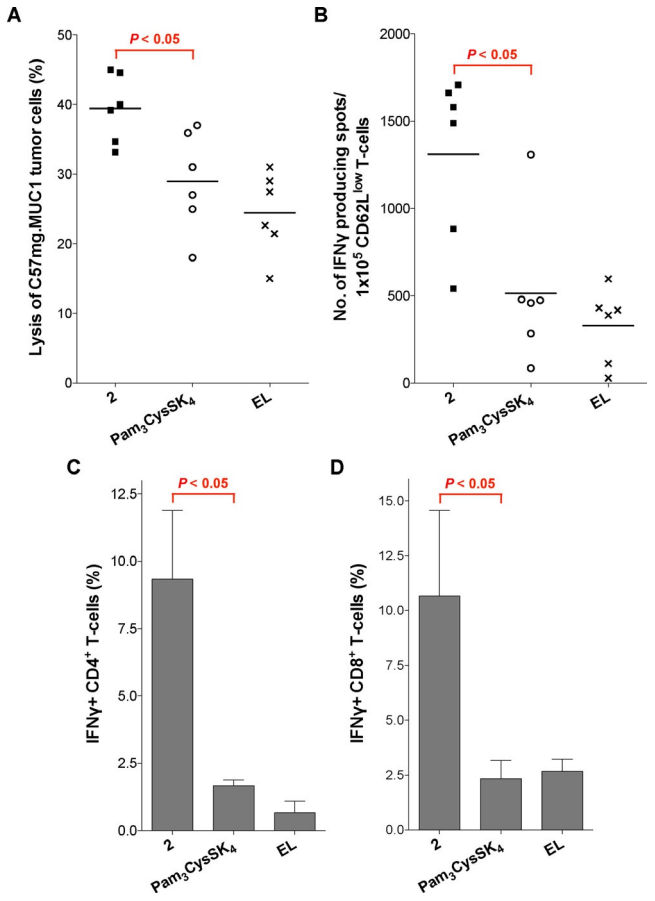


Figure 1. A) Induction of ADCC with C57mg.MUC1 tumor cells. B) Induction of cytotoxic CD62L^{low} T-cell response by analyzing MUC1-specific IFNγ spot formation without in vitro stimulation. Each data point represents an individual mouse, and horizontal lines indicate the mean for the group of mice. ICC staining for the presence of C) CD4$^+$IFNγ$^+$ and D) CD8$^+$IFNγ$^+$ T-cells. See Figure S3 for representative flow cytometry plots. Data are shown as mean ± SEM ($n = 3$) for vaccine candidate **2** (Pam_3CysSK_4 APGSTAPPAHGVT-SAPDT(*O*-GalNAc)RPAP) and controls Pam_3CysSK_4 and empty liposomes (EL).

pated, both CD4$^+$ and CD8$^+$ T-cells derived from mice immunized with multicomponent vaccine **2** were responsive to the MUC1 epitope.

Collectively, our data demonstrated that vaccine candidate 2, which is devoid of any artificial linkers or exogenous helper T-epitopes, can activate T-cells and elicit robust cytotoxic IgG antibody responses. Previously, a number of synthetic MUC1 vaccines have been reported containing a full-length tandem repeating unit.[8b,e,h,n,18] Although these vaccines were examined in wild-type mice that did not require breaking immune tolerance, they elicited relatively low titers of IgG antibodies. The modest immune activation was probably due to the fact that these vaccine candidates contain a MUC1 tandem repeat (e.g., AHGVTSAPDTRPAPGSTAPP) that does not embed the full putative helper T-epitope. In addition, some of these immunogens were glycosylated at all serine and threonine moieties of the MUC1 tandem repeat, which might compromise proteolytic processing to provide epitopes that can be presented by MHCI or MHCII.[15,19] Linkers employed to connect the Pam_3CysSK_4 moiety with the MUC1 glycopeptide could also interfere with proteolytic processing. Thus, careful selection of the MUC1 tandem repeating unit and proper glycosylation are likely critical determinates of a multicomponent vaccine that can activate each arm of the immune system. It is the expectation that a MUC1-based vaccine that contains an endogenous helper T-epitope will promote induction of cognate CD4$^+$ T-cells that could provide critical tumor-specific help intratumorally during the effector stage of tumor rejection and potentially aid in the generation of sustained immunological memory.[13a,20] Future studies will focus on establishing the optimal glycosylation pattern of the MUC1 tandem repeating unit to elicit the most tumor-relevant immune responses.

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Conflict of Interest

G.-J.B. is the founder of Viamune, a company that focuses on MUC1 cancer vaccine development.

Keywords: cancer · endogenous helper · glycopeptide · multicomponent vaccine · T-epitope

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