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Towards a Fully Synthetic Carbohydrate-Based Anticancer Vaccine: Synthesis and Immunological Evaluation of a Lipidated Glycopeptide Containing the Tumor-Associated Tn Antigen**

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Dedicated to Professor Steven V. Ley on the occasion of his 60th birthday

A common feature of oncogenic transformed cells is the overexpression of oligosaccharides, such as Globo-H, Lewis^Y, and Tn antigens.[1–4] Numerous studies have shown that this abnormal glycosylation can promote metastasis,[5] and hence its expression is strongly correlated with poor survival rates of cancer patients.

Several elegant studies have exploited the differential expression of tumor-associated carbohydrates for the development of cancer vaccines.[6,7] The inability of carbohydrates to activate helper T lymphocytes has complicated, however, their use as vaccines.[8] For most immunogens, including carbohydrates, the production of antibodies depends on the cooperative interaction of two types of lymphocytes, B cells and helper T cells.[9] Saccharides alone cannot activate helper T cells and therefore have a limited immunogenicity. The formation of low-affinity IgM antibodies and the absence of IgG antibodies manifest this limited immunogenicity.

To overcome the T cell independent properties of carbohydrates, past research has focused on the conjugation of saccharides to a foreign carrier protein (e.g. Keyhole Limpet Hemocyanin (KLH) or detoxified tetanus toxoid).[8,9] In this approach, the carrier protein enhances the presentation of the carbohydrate to the immune system and provides T epitopes (peptide fragments of 12–15 amino acids) that can activate helper T cells.

However, the conjugation of carbohydrates to a carrier protein poses several problems. In general, the conjugation chemistry is difficult to control, resulting in conjugates with ambiguities in composition and structure which may affect the reproducibility of an immune response.[10] Additionally, the foreign carrier protein can elicit a strong B cell response, which may lead to the suppression of an antibody response against the carbohydrate epitope. The latter is a greater

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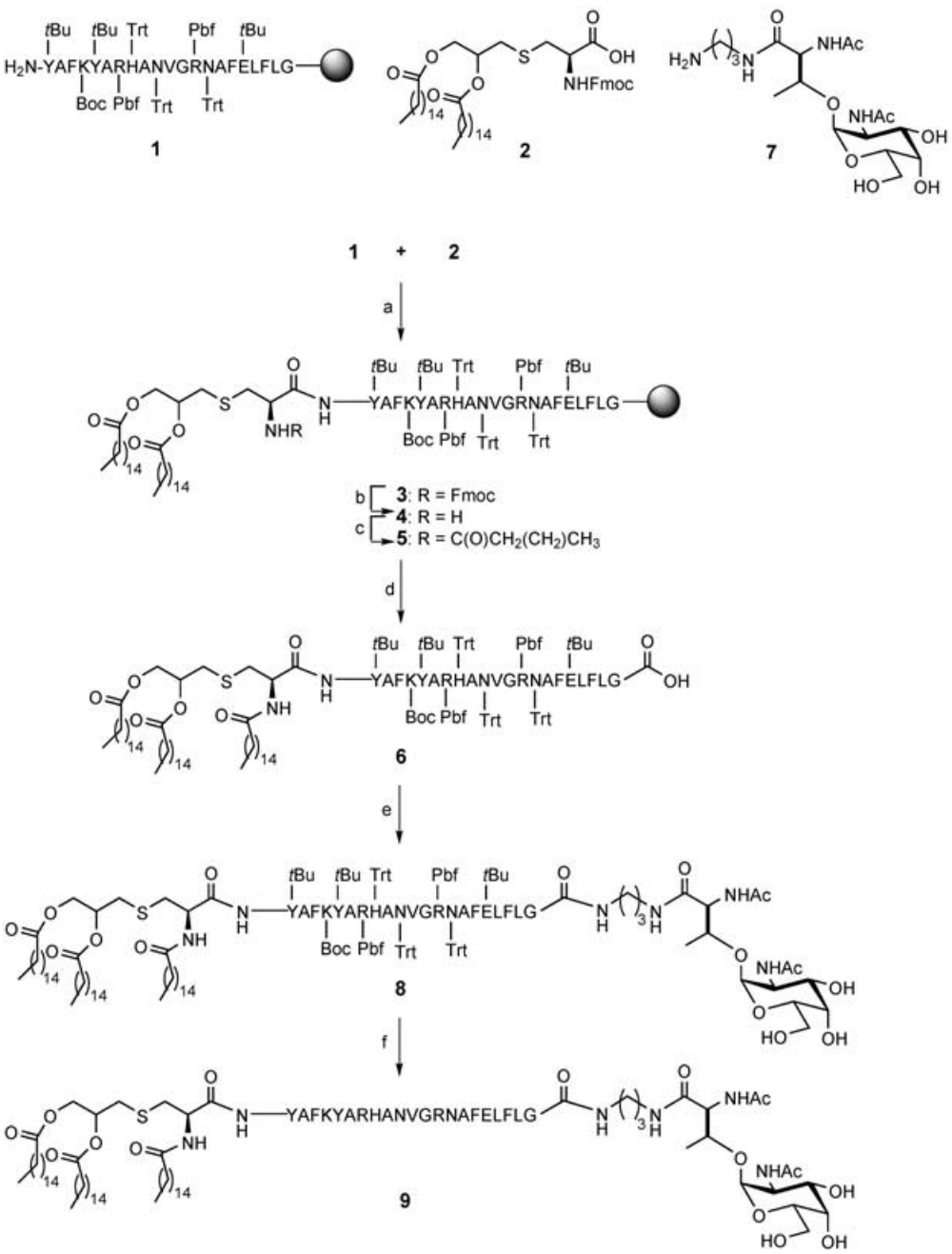
problem when self-antigens are employed, such as tumor-associated carbohydrates. Also linkers for the conjugation of carbohydrates to proteins can be immunogenic, leading to epitope suppression.[11] Not surprisingly, several clinical trials with carbohydrate–protein conjugate cancer vaccines failed to induce sufficiently strong helper T cell responses in all patients.[7,12] Therefore, alternative strategies need to be developed for the presentation of tumor-associated carbohydrate epitopes that will result in a more efficient class switch to IgG antibodies.[13–18]

Herein, we report the synthesis and immunological evaluation of a structurally well-defined, fully synthetic anticancer vaccine candidate **9** that constitutes the minimal structural features required for a focused and effective T cell dependent immune response. The vaccine candidate is composed of the tumor-associated Tn antigen, the peptide T epitope YAFKYARHANVGRNAFELFL (YAF), and the lipopeptide *S*-[(*R*)-2,3-dipalmitoyloxy-propyl]-*N*-palmitoyl-(*R*)-cysteine (Pam_3Cys). The Tn antigen, which will serve as a B epitope, is overexpressed on the surface of human epithelial tumor cells of the breast, colon, and prostate.[3] This antigen is not present on normal cells, which thus renders it an excellent target for immunotherapy.[3,13,18] To overcome the T cell independent properties of the carbohydrate antigen, the YAF peptide was incorporated. This 20-amino acid peptide sequence is derived from an outer-membrane protein of *Neisseria meningitides* and has been identified as an MHC class II restricted site for human T cells.[19] It was envisaged that this helper T cell epitope would induce a T cell dependent immune response that results in the production of IgG antibodies against the Tn antigen. The combined B cell and helper T cell epitope lacks the ability to provide appropriate "danger signals"[20] for dendritic cell maturation. Therefore, the lipopeptide Pam_3Cys, which is derived from the immunologically active N-terminal sequence of the principal lipoprotein of *Escherichia coli*,[21] was incorporated. This lipopeptide has been recognized as a powerful immunoadjuvant,[22] and recent studies have shown that it exerts its activity through the interaction with Toll-like receptor 2 (TLR-2).[23] This interaction results in the production of pro-inflammatory cytokines and chemokines, which, in turn, stimulate antigen-presenting cells (APCs) and thus initiate development and activation of helper T cells.[24] The lipopeptide also facilitates the incorporation of the antigen into liposomes. Liposomes have attracted interest as vectors in vaccine design[25] owing to their low intrinsic immunogenicity, thus, avoiding undesirable carrier-induced immune responses.

The synthesis of target compound **9** requires a highly convergent synthetic strategy which employs chemical manipulations that are compatible with the presence of a carbohydrate, peptide, and lipid moieties. It was envisaged that **9** could be prepared from spacer-containing Tn antigen **7**, polymer-bound peptide **1**, and *S*-[2,3-bis(palmitoyloxy)-propyl]-*N*-Fmoc-Cys ($Pam_2FmocCys$, **2**;[26] Fmoc = 9-fluorenylmethoxycarbonyl). The resin-bound peptide **1** was assembled by automated solid-phase peptide synthesis using Fmoc-protected amino acids in combination with the extremely acid-sensitive HMPB-MBHA (HMPB = 4-(4-hydroxymethyl-3-methoxyphenoxy)butyryl; MBHA = *p*-methylbenzhydrylamine) resin and 2-(1*H*-benzotriazol-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole (HBTU/HOBt)[27] as activators (Scheme 1). The HMPB-MBHA resin was selected because it allows the cleavage of a compound from the resin without concomitant removal of side-chain protecting groups. This feature was important because side-chain functional groups of aspartic acid, glutamic acid, and lysine would otherwise interfere with the incorporation of the Tn antigen derivative **7**. Next, the $Pam_2FmocCys$ derivative (**2**) was coupled to the N-terminal amine of peptide **1** using PyBOP[28] and HOBt in the presence of DIPEA in a mixture of DMF and dichloromethane to give the resin-bound lipopeptide **3**. The Fmoc group of **3** was removed under standard conditions, and the free amine of the resulting compound **4** was coupled with palmitic acid in the presence of PyBOP and HOBt to give the fully protected and resin-bound lipopeptide **5**. The amine of the Pam_2Cys moiety was palmitoylated after coupling with **1** to avoid racemization of the cysteine moiety.[26] Cleavage of compound **5** from the resin was achieved with 2% TFA in dichloromethane followed by the immediate neutralization with 5% pyridine in methanol. After purification by LH-20 size-exclusion chromatography, the C-terminal carboxylic acid of lipopeptide **6** was coupled with the amine of Tn derivative **7**, employing DIC/HOAt/DIPEA[29] as coupling reagents, to give, after purification by Sephadex LH-20 size-exclusion chromatography, fully protected lipidated glycopeptide **8** in 79% yield. MALDI-TOF mass spectrometry revealed signals at $m/z = 5239.6$ and $m/z = 5263.0$, which correspond to $[M+H]^+$ and $[M+Na]^+$ ions, respectively. Finally, the side-chain protecting groups of **8** were removed by treatment with 95% TFA in water using EDT as a scavenger. The alternative use of triisopropylsilane resulted in the formation of unidentified byproducts. The target compound **9** was purified by size-exclusion chromatography followed by reverse-phase HPLC using a Synchropak C4 column. Analysis of **9** by MALDI-TOF mass spectrometry revealed a signal at $m/z = 3760.3$ corresponding to $[M+Na]^+$.

Next, compound **9** was incorporated into phospholipid-based liposomes. Thus, after hydration of a lipid film that contained **9**, cholesterol, phosphatidylcholine, and phosphatidylethanolamine, small unilamellar vesicles (SUVs) were prepared by extrusion through 100-nm Nuclepore polycarbonate membranes. Transmission electron microscopy (TEM) by negative stain confirmed that the liposomes were uniformly sized with an expected diameter of approximately 100 nm (Figure 1). The liposome preparations were analyzed for *N*-acetyl galactosamine (GalNAc) content by hydrolysis with TFA, followed by quantification with anion-exchange chromatography at high pH values. Concentrations of approximately $30\ \mu g\,mL^{-1}$ of GalNAc were determined which corresponded to an incorporation of **9** of approximately 10%.

Groups of five female BALB/c mice were immunized subcutaneously with freshly prepared liposomes containing 0.6 μg carbohydrate at weekly intervals. To explore the adjuvant properties of the built-in lipopeptide Pam_3Cys, the antigen-containing liposomes were administered with or without the potent saponin immunoadjuvant QS-21 (Anti-



Scheme 1. a) PyBOP, HOBt, DIPEA, DMF/CH_2Cl_2 (5:1); b) piperidine/DMF (1:5); c) $CH_3(CH_2)_{14}COOH$, PyBOP, HOBt, DMF/CH_2Cl_2 (1:5); d) 2% TFA in CH_2Cl_2; e) **7**, DIC, HOAt, DIPEA, DMF/CH_2Cl_2 (2:1), 79%; f) TFA/H_2O/EDT (95:2.5:2.5), 79%. Boc = *tert*-butyloxycarbonyl; Trt = trityl; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DIPEA = diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; TFA = trifluoroacetic acid; DIC = diisopropylcarbodiimide; HOAt = 1-hydroxy-7-azabenzotriazole; EDT = 1,2-ethanedithiol. Ratios for solvent mixtures are indicated as v/v.

genics Inc., Lexington, MA). Anti-Tn antibody titres were determined by coating microtitre plates with a BSA–Tn conjugate (BSA = bovine serum albumin) and detection was accomplished with anti-mouse IgM or IgG antibodies labeled with alkaline phosphatase. As can be seen in Table 1, the mice that were immunized with the liposome preparations elicited IgM and IgG antibodies against the Tn antigen (Table 1, entries 1 and 2). The presence of IgG antibodies indicated that the helper T epitope peptide of **9** had activated helper T lymphocytes. Furthermore, the observation that IgG antibodies were raised by mice which were only immunized with liposomes (group 1) indicated that the built-in adjuvant Pam_3Cys had triggered appropriate signals for the maturation of dendritic cells and their subsequent activation of helper T cells. However, the mice which received the liposomes in combination with QS-21 (group 2), elicited higher titres of anti-Tn antibodies. This stronger immune response may be due to a shift from a mixed Th1/Th2 to a Th1 response.[30]

The results presented herein provide, for the first time, a proof of principle for the use of lipidated glycopeptides as minimal subunit vaccines. Previous immunizations with a saccharide coupled to an immunoadjuvant such as Pam_3Cys resulted in no or very low titres of IgG antibodies[15,31,32] demonstrating that the incorporation of a peptide T epitope[16,17] is critical for a class switch to IgG antibodies.

It is to be expected that several improvements can be made to the tricomponent vaccine candidate presented here. For example, it has been found that a clustered presentation of the Tn antigen is a more appropriate mimetic of mucins, and hence antibodies raised against this structure recognize better Tn antigens expressed on cancer cells.[33–36] The Th epitope employed in this study is known to be a MHC class II restricted epitope for humans. Thus, a more efficient class switch to IgG antibodies may be expected when a murine Th epitope is employed. On the other hand, compound **9** is a more appropriate vaccine candidate for use in humans. A recent report indicated that Pam_2Cys is a more potent immunoadjuvant than Pam_3Cys.[37] It has also been suggested that the Pam_2Cys adjuvant has

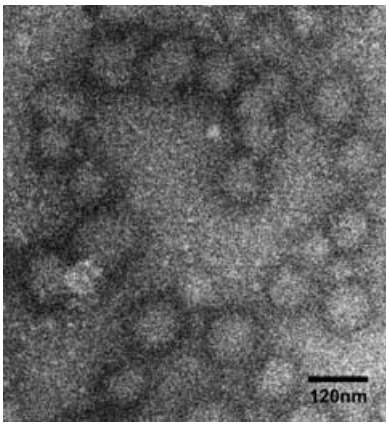


Figure 1. Negative-stain transmission electron micrograph of the liposome preparation.

Table 1: ELISA anti-Tn antibody titres[a] after four immunizations with the glycolipopeptide/liposome formulation.

Entry	Group	IgM titres	IgG titres
1	1) Pam_3Cys-YAF-Tn	250	1410
2	2) Pam_3Cys-YAF-Tn + QS-21	170	2675

[a] ELISA plates were coated with a BSA-BrAc-Tn conjugate (BrAc = 3-(bromoacetamido)propionate). All titres are medians for a group of five mice. Titres were determined by regression analysis, plotting $\log_{10}$-(dilution) versus the absorbance. The titres were calculated to be the highest dilution that gave 0.1 or higher than the absorbance of normal saline mouse sera diluted 1:100.

improved solubility properties,[38] which is a problematic feature of compound **9**. Studies addressing these issues are ongoing.

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