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Synthesis and immunological evaluation of an antitumor neoglycopeptide vaccine bearing a novel homoserine Tn antigen

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Abstract—As part of our program on Tn-specific anti-tumor immunotherapy, our aim was to vary the nature of the aglyconic part of the tumor-associated Tn antigen (α-D-GalNAc-Ser/Thr). This report describes the synthesis of Fmoc-hSer-(α-D-GalNAc)-OH (**4**) in 19% overall yield from protected aspartic acid. The building block **4** was incorporated as trimeric clusters into a glycopeptide vaccine [MAG:Tn(hSer)3-PV], using solid-phase peptide synthesis. When injected in mice, the resulting MAG induces a strong antibody response, which recognizes native tumor-associated antigens (TAA) at the surface of human tumor cells. This approach may be extended to the use of other nonnatural TAA in order to improve half-life of synthetic anti-cancer vaccines.

Over the last years, numerous carbohydrate tumor-associated antigens (TAA) have been identified and used as potential targets for anti-cancer therapy: Tn, T, sialyl-Tn, Lewis antigens, and glycolipid-derived antigens (KH-1, globo H, GM2, ...).[1] To generate an efficient immune response, these TAA are usually linked to an immunogenic carrier protein.

To overcome the drawbacks associated with the protein conjugates (uncertainty in both composition and structure, low hapten density, irrelevant antibody production), we designed an efficient synthetic immunogen, the multiple antigen glycopeptide (MAG).[2–4] We prepared MAG vaccines displaying the Tn antigen (α-D-GalNAc-Ser/Thr) as the carbohydrate TAA. This antigen is overexpressed on epithelial tumors and is associated with many cancers including breast, prostate, lung, and pancreatic cancers.[5,6] To mimic the clustered motif encountered in vivo, we chose a tri-Tn mucin-like glycotope recognized by the tumor specific MLS-128 monoclonal antibody:[7] Ser(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr(α-D-GalNAc). The resulting MAG vaccine MAG:Tn3-PV, based on a dendrimeric lysine core carrying four copies of a $CD4^+$ T-cell peptide epitope together with the tri-Tn glycotope, is highly immunogenic in mice and afforded good protection in prophylactic and therapeutic vaccinations against the development of Tn-expressing tumor cells.[4] The anti-Tn antibody response is abolished when mice were depleted of CD4 T cells in vivo, showing the absolute requirement of the PV specific CD4 T-cell response.[4]

Preliminary results of our laboratory show that the amino-acid carrying the Tn antigen (Ser or Thr residues) contributes to the antibody recognition. Indeed, by varying the tri-Tn glycotope structure in the MAG, we obtained different monoclonal antibodies specific for the Ser(α-D-GalNAc)-Ser(α-D-GalNAc)-Ser(α-D-GalNAc)

Abbreviations: AAA, amino acid analysis; biot, biotine; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; ESMS, electrospray mass spectrometry; FACS, fluorescent activated cell sorter; hSer, homoserine; MAP, multiple antigenic peptide; MAG, multiple antigenic glycopeptide; PV, poliovirus; RP-HPLC, reverse-phase high-performance liquid chromatography; Ser, serine; SPPS, solid-phase peptide synthesis; TAA, tumor-associated antigen.

Keywords: MAG: multiple antigenic glycopeptide; Tn antigen; Homoserine; Immunogenicity; Antibodies.

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sequence or the Ser(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr(α-D-GalNAc) sequence (R. Lo Man and coll., unpublished results).

Approaches involving Tn antigen analogues have only been reported by a few laboratories: a neoglycopeptide with an analogue bearing a *C*-glycosyl linkage[8] and a protein glycoconjugate bearing a longer *O*-aliphatic aglycone.[9] In both cases the Tn analogue was incorporated as a monomer. Interestingly, the later vaccine construct was more antigenic than the natural Tn-based conjugate.[10]

In order to vary the nature of the aglyconic part of the Tn structure, we used the homo-serine (hSer) as the amino-acid carrier for the GalNAc residue. hSer allows to increase the distance of the carbohydrate residues from the peptide chain by one carbon atom as compared to its serine analog. This nonnatural amino-acid has already been included in a linear glycopeptide bearing a single tumor-associated disaccharide,[11] but, to our knowledge, the immunogenicity of this construct was not analyzed.

In the present paper, we prepared a new MAG bearing several α-D-GalNAc residues displayed as trimeric clusters on a hSer-hSer-hSer motif [MAG:Tn(hSer)3-PV] and we show that the injection of this neoglycopeptide in mice raises antibodies, which are able to recognize native TAA at the surface of human tumor cells.

Preparation of glycosylated building block Fmoc-hSer-(α-D-GalNac)-OH **4** *(Scheme 1):* For the synthesis of *N*-α-Fmoc-L-homoserine-α-*t*-butyl ester (Fmoc-hSer-O*t*Bu **1**), commercially available *N*-α-Fmoc-L-aspartic acid-α-*t*-butyl ester was reduced to the corresponding alcohol.[12] This method avoids the formation of the Fmoc-γ-lactone occurring during the Fmoc-protection of hSer[11] and allows to obtain in a single step the Fmoc-hSer-O*t*Bu **1** (65%) appropriately protected for the glycosylation reaction.

The 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl chloride[13] was prepared in three steps starting from the tri-*O*-acetyl-D-galactal.[14] It was then added to the Fmoc-hSer-O*t*Bu **1** for the Koenigs–Knorr condensation.[15] Although trimethylsilyl trifluoromethane sulfonate (AgOTf)[16] was found to be the best catalyst for the condensation of serine derivatives, in our hands, the catalysts silver carbonate (Ag_2CO_3)/silver perchlorate ($AgClO_4$)[17] afforded the α-anomer in a better yield. Separation of the two anomers by flash chromatography was achieved after reduction and acetylation of the azido group (overall yield 88% based on **1**, α/β: 76/24).

The final deprotection of the *t*-butyl ester and the *O*-acetyl groups of **3**[17] afforded the glycosylated hSer building block **4** appropriately protected for the peptide synthesis with 19% overall yield.[18]

Solid-phase synthesis of MAG:Tn(hSer)3-PV (Fig. 1): MAG:Tn(hSer)3-PV was assembled by the conventional solid-phase peptide methodology (Wang resin) using Fmoc chemistry as described previously.[2,4] The protected amino acids were incorporated manually into the peptide sequence using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBT)/diisopropylethylamine (DIEA) as the coupling reagent.[19,20] Fmoc protection was removed with 20% piperidine in DMF.

Three glycosylated building blocks [Fmoc-hSer(α-D-GalNAc)-OH **4**] were incorporated successively as their pentafluorophenyl (Pfp) esters in the presence of HOBT.[21] The Pfp esters were prepared by addition of

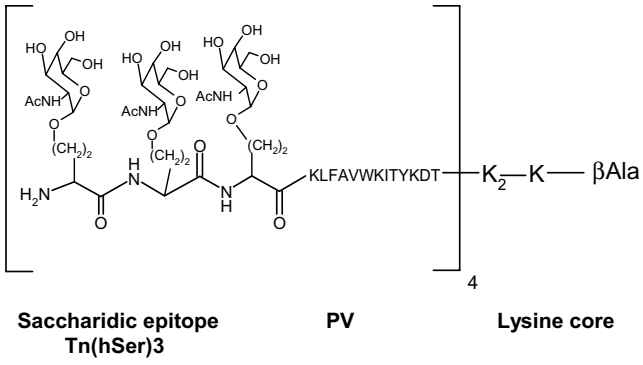
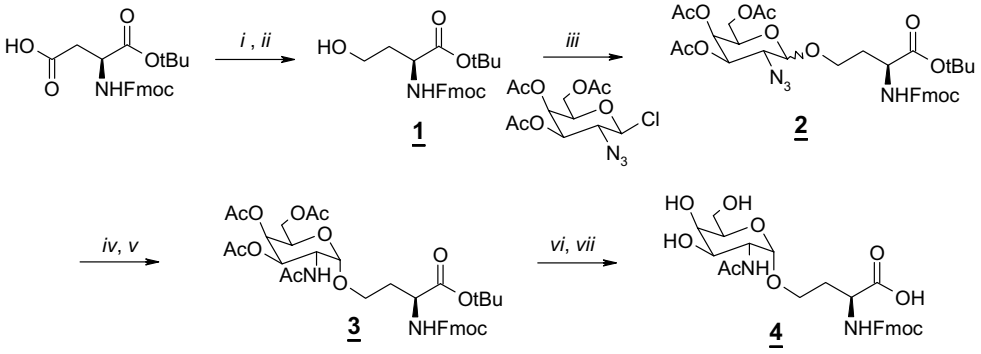


Figure 1. Schematic representation of MAG:Tn(hSer)3-PV.



Scheme 1. Preparation of glycosylated homoserine (Fmoc-hSer-(α-D-GalNac)-OH). Reagents, conditions, and yields: (i) EtOCOCl, Et_3N, THF, 1 h, −10 °C; (ii) $NaBH_4$, H_2O, 65% (two steps); (iii) Ag_2CO_3, $AgClO_4$, CH_2Cl_2, toluene; (iv) $NiCl_2$, H_3BO_3, $NaBH_4$, EtOH; (v) Ac_2O, 67% (three steps); (vi) HCOOH, 98%; (vii) MeONa, MeOH, 46%.

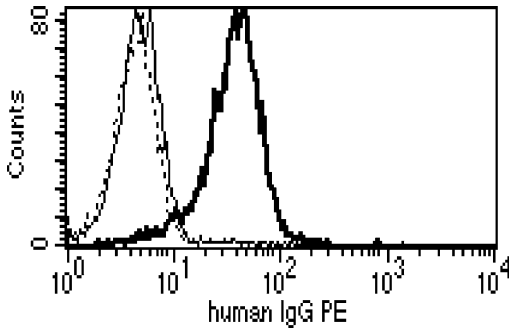


Figure 2. Recognition of a human tumor cell line by sera from MAG:Tn(hSer)3-PV-primed mice. Mice were injected on days 0 and 21 with MAG:Tn(hSer)3-PV (bold line) or with MAP:PV devoided of Tn (thin line) in alum, and sera collected on day 28 were analyzed by FACS for recognition of Tn-positive Jurkat cells. Dotted line corresponds to unstained cells.

1,3-diisopropylcarbodiimide to the glycosylated amino acid and pentafluorophenol in dry dichloromethane;[22] they were used directly after concentration without purification. The products were cleaved from the resin with aqueous trifluoroacetic acid (TFA), triisopropylsilane, H_2O, phenol.

The MAG (Fig. 1) was purified by reverse-phase high-performance liquid chromatography with gradient performed with water (0.1% TFA)/acetonitrile on a C18 column and it was characterized by amino acid analysis (AAA) and electrospray mass spectrometry (ESMS).[23]

In mice, MAG:Tn(hSer)3-PV induces specific antibodies which recognize a human tumor cell line (Fig. 2): We analyzed the immunogenicity of MAG:Tn(hSer)3-PV in BALB/c mice. Sera from immunized mice were tested by enzyme-linked immunosorbent assay (ELISA) against a biotinylated synthetic Tn(hSer) cluster [Tn(hSer)3-G6K(biot)G] coated on streptavidin plates.[24] The non-glycosylated analogue was used as a control for background reactivity. Immunization with the MAG induced specific IgG antibodies, which recognized the Tn(hSer)3 cluster (data not shown).

In a previous study, we demonstrated that anti-Tn IgG antibody titers obtained by immunization with a linear glycopeptide bearing three D-serine residues were similar to those obtained with a glycopeptide containing three L-serine, as measured by FACS using human tumor cells.[25] Indeed this method gives a more accurate view of the 'natural' display of TAA at the cell surface for antibody recognition.

To ensure that the antibodies induced by the MAG:Tn(hSer)3-PV were able to recognize the native Tn antigen on tumor cells, we analyzed the binding of mouse sera to human Jurkat T-lymphoma cells that express the Tn antigen. Figure 2 shows that these cells were recognized by the MAG-induced antibodies, showing the biological relevancy of the immune response.

In conclusion, we describe (i) an efficient route for the synthesis of a glycosylated homoserine building block as a Tn antigen analogue and (ii) its incorporation in an anti-tumor vaccine neoglycopeptide by SPPS. We also show that the immune response induced in mice by this vaccine is biologically relevant since the resulting antibodies recognize a human tumor cell line.

These results open new perspectives for the rational design of synthetic anti-cancer vaccines with longer half-life. Indeed the incorporation of nonnatural Tn analogues in glycopeptide vaccines should prevent the in vivo degradation of the natural glycosyl-serine linkage.

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(m, 1H, CH hSer), 3.97 (m, 1H, H-2), 3.66 (br d, 1H, H-4), 3.55 (m, H-3, H-5), 3.44, 3.40 (m, H-6, H-6′), 3.55, 3.24 (CH_2O hSer), 1.94 (m, 1H, CH_2CH), 1.77 (m, 4H, CH_2CH, CH_3 Ac) ; ^{13}C NMR ($(CD_3)_2SO$, 100 MHz): δ 174.76, 170.64 (CONH, COOH), 156.92 (OCONH), 144.72, 144.64, 141.57, 141.55 (C Phe), 128.50, 127.95, 126.11, 126.06, 120.96 (CH Phe), 98.50 (C-1), 72.14, 68.96, 68.73 (C-3, C-4, C-5), 66.48 (CH_2 Fmoc), 64.65 (CH_2O), 61.44 (C-6), 52.00 (CH hSer), 50.60 (C-2), 47.52 (CH Fmoc), 31.79 (CH_2CH), 23.54 (CH_3 Ac). FABMS for $C_{27}H_{32}N_2O_{10}$: (calcd 544.21) m/z 567.3 $[M+Na]^+$.

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