Glycopeptide Vaccines

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Variation of the Glycosylation Pattern in MUC1 Glycopeptide BSA Vaccines and Its Influence on the Immune Response**

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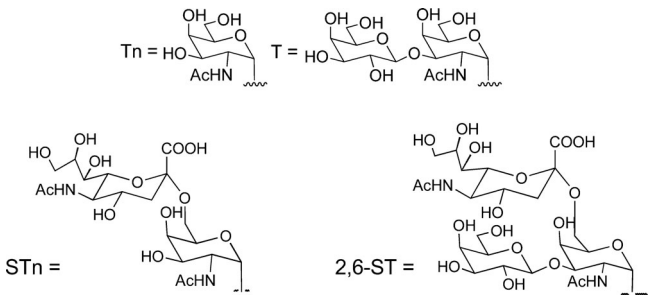
Dedicated to Professor Joachim Thiem on the occasion of his 70th birthday

Because of its overexpression on almost all types of epithelial tumor tissues, the tumor-associated mucin MUC1 is an attractive target antigen for cancer immunotherapy.[1] A number of research groups are interested in the development of antitumor vaccines based on this mucin.[2] Compared with MUC1 on normal cells, the glycan profile of tumor-associated MUC1 is characteristically changed, in particular, in the extracellular domain, which consists of a variable number of tandem repeat sequences HGVTSAPDTRPAPGSTAPPA containing five potential O-glycosylation sites (T4, S5, T9, S15, and T16).[3] As a resulting of downregulation of a glucosaminyl transferase and concomitant overexpression of sialyl transferases,[4] MUC1 on tumor cells carries short, often prematurely sialylated glycan side chains.[2d] The tumor-associated carbohydrate antigens comprise the Thomsen–Friedenreich antigen (T antigen),[5] its precursor (Tn antigen), and their respective sialylated derivatives STn and 2,6-ST.[6] Because of the truncated, short saccharide side chains, peptide epitopes within the protein backbone of tumor-associated MUC1 are uncovered.[7] These aberrant glycopeptides are considered promising target structures for the development of antitumor vaccines.



Owing to the biological microheterogeneity of glycoproteins, MUC1 isolated from tumor cell membranes is not suitable for vaccination.[1,2e] Synthetic glycopeptides of the MUC1 tandem repeat sequence which contain structurally defined saccharides should facilitate the induction of sufficiently tumor-selective immune responses. As recently discussed,[8] the glycosylation of serine and/or threonine in the MUC1 tandem repeat sequence can distinctly influence the conformation of the peptide backbone[9] and the immunogenicity of the tumor-associated glycopeptides. Therefore, the influence of the different saccharide attachment sites within the MUC1 glycopeptide vaccine on the immune responses is of particular interest.

In a previous study,[8] synthetic MUC1 glycopeptides bearing Tn and/or T antigens at serine 15 (S15) and threonine 9 (T9) of the tandem repeat sequence were conjugated to bovine serum albumin (BSA) as the carrier protein (Scheme 1). Immunization of Balb/c mice with these vaccines revealed that the glycosylation of the tandem repeat sequence with either the Tn or the T antigen at position T9, which belongs to the immunodominant PDTRP epitope, enhanced the immune response. These results prompted us to synthesize MUC1 tandem repeat peptides bearing the tumor-associated sialylated saccharide antigens STn or 2,6-ST at position S15, while threonine T9 is linked to Tn or T antigen or is not glycosylated. As sialyltransferases have been found to be strongly overexpressed on epithelial tumor cells,[10] the investigation of MUC1 vaccines with sialylated saccharide antigens is considered of particular interest.

The glycopeptides were covalently linked to BSA. The resulting vaccines were used for immunization of Balb/c mice. The antibody titers of the induced antisera were determined by enzyme-linked immunosorbent assay (ELISA). In addition, the isotypes of the induced antibodies were examined. The binding of the induced sera to the MUC1 expressed on human MCF-7 breast tumor cells was examined by flow cytometry (FACS analysis).

The microwave-supported solid-phase synthesis[8] of the MUC1 glycopeptides containing the STn or the 2,6-ST antigen at site S15 and Tn or T antigen at site T9 was performed starting from a 2-chlorotrityl resin preloaded with Fmoc-alanine (Scheme 2). The peptide coupling was carried out with Fmoc amino acids (6 equiv) using HBTU/HOBt. The more reactive HATU/HOAt was used for coupling of the

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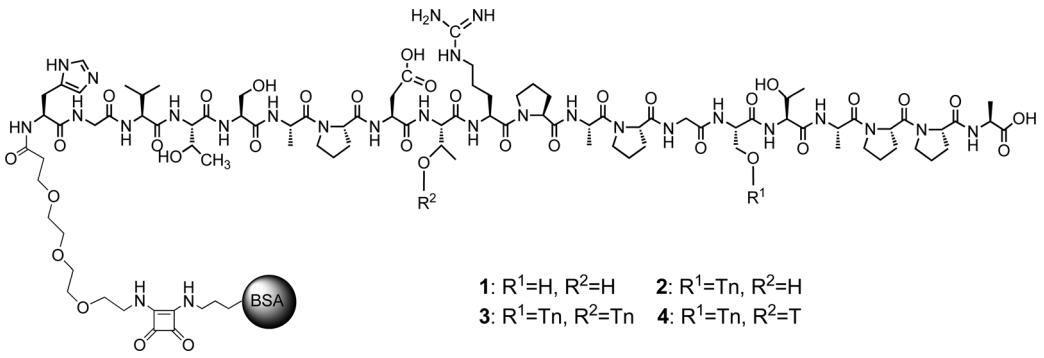
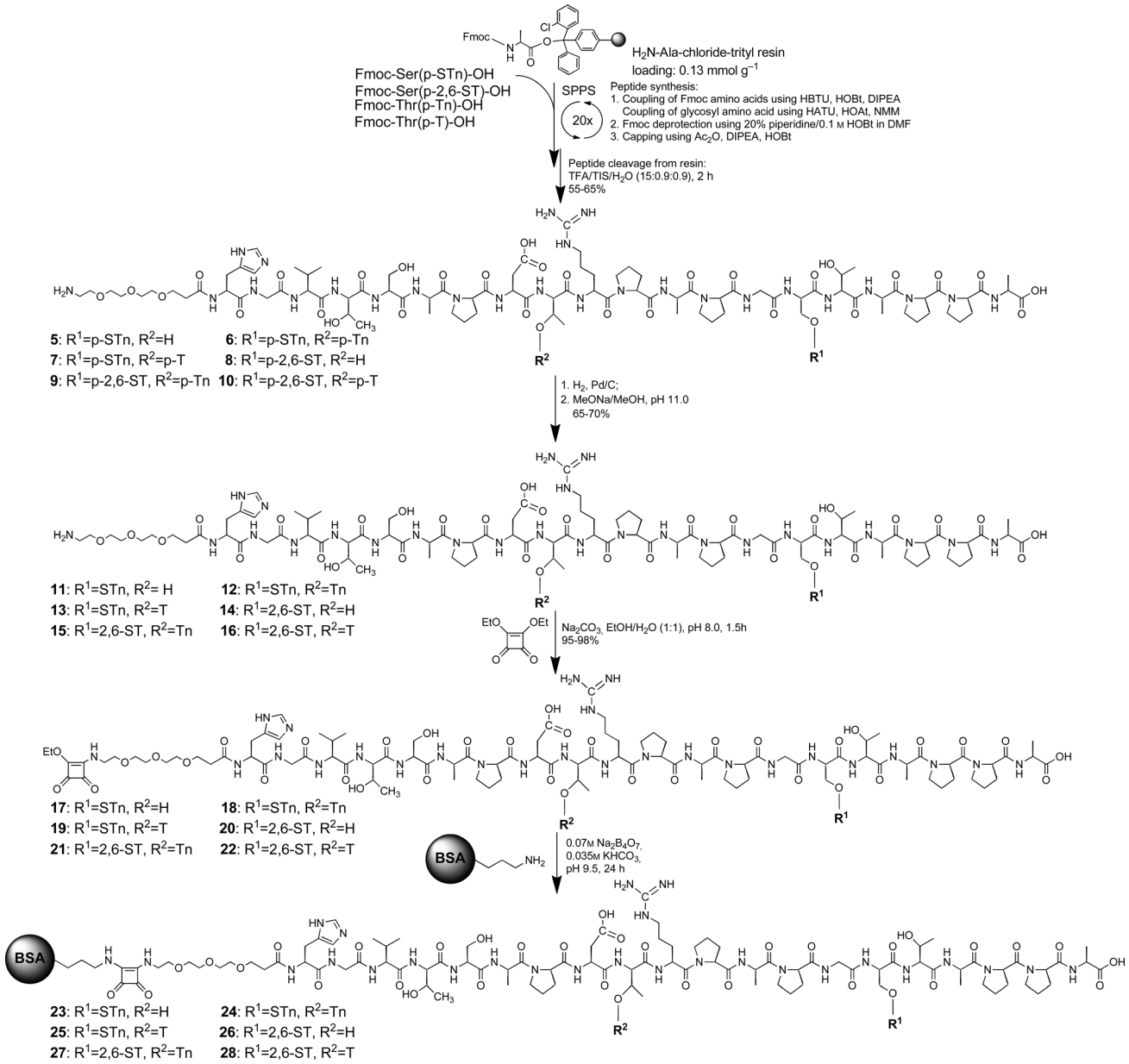


Scheme 1. Structure of vaccines consisting of MUC1 glycopeptides bearing Tn and T antigens conjugated to BSA.

Fmoc *O*-glycosyl amino acids.[11] Each glycopeptide was extended at the N terminus with a triethylene glycol spacer amino acid.[2c–g]

After completion of the synthesis the crude glycopeptides were detached from the resin using TFA/TIS/H_2O and concomitantly all acid-sensitive side-



Scheme 2. Solid-phase synthesis of the MUC1 glycopeptides and their conjugation to BSA. Antigen abbreviations preceded by the letter p indicate the completely O-acetylated form of the antigen SPPS = solid-phase peptide synthesis, Fmoc = fluorenyl-9-methoxycarbonyl, HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = *N*-hydroxy-7-azabenzotriazole, NMM = *N*-methylmorpholine, TFA = trifluoroactic acid, TIS = triisopropylsilane.

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chain protecting groups of the amino acids were removed. After purification by preparative HPLC on a C-18 column, the glycopeptides **5**–**10** still protected in the glycan portions were isolated in yields of 55–65%. The benzyl ester and benzyl ether groups within the saccharide moieties were removed by hydrogenation in the presence of Pd/C (5%). The *O*-acetyl groups were removed by treatment with a NaOMe/MeOH solution (pH 11.0). The deprotected glycopeptides **11**–**16** were purified by preparative HPLC and isolated in yields of 65–70%. The terminal amino group on the spacer was reacted with diethyl squarate[12] in EtOH/H_2O at pH 8.0 resulting in the squaric acid monoamides **17**–**22** of the glycopeptides. These glycopeptides were dissolved together with BSA in a buffer solution at pH 9.5 to afford the glycopeptide vaccines **23**–**28**.[2d] The loading of the glycopeptide-BSA conjugates (on average nine molecules of glycopeptide per molecule of BSA) was determined by MALDI-TOF mass spectrometry (see the Supporting Information).

To evaluate the immunological properties of the glycopeptide–BSA conjugates, 10 μg of each of these synthetic vaccines in combination with an adjuvant were subcutaneously injected into Balb/c mice according to the reported procedures.[2e] Complete Freund's adjuvant was used for the first immunization, incomplete Freund's adjuvant for each subsequent booster. One week after the third immunization the sera of the mice were analyzed by ELISA for detection of the induced antibodies. The microtiter plates were coated with unconjugated glycopeptides **11**–**16** dissolved in 0.1M $NaHCO_3$ (pH 9.6).[2a]

Strong immune responses were observed for all applied vaccines. The titers determined in ELISA tests (Figure 1) amounted to approximately 6000 to 30000; here, the titers were defined as the dilution yielding the half-intensity optical density (inflection point of the curve). The corresponding end-point titers varied between 51200 and 204800. These results were unexpected and surprising as BSA vaccines containing incomplete MUC1 tandem repeat sequences with sialylated saccharide antigens had shown low immunogenicity in earlier investigations.[2d] In addition, a fully synthetic MUC1 glycopeptide–OVA T-cell epitope vaccine carrying an STn antigen at T4 and a Tn antigen at T9 in the PDTRP domain exhibited no immunogenicity at all.[2g]

The determination of the antibody isotypes induced by non-sialylated vaccines **1**–**4** and by sialylated vaccines **23**–**28** were carried out using isotype-selective secondary antibodies. To this end, induced sera were diluted (1:1000) for the ELISA measurements, which indicated that the IgG_1 isotype is predominant in each case, but IgM antibodies are also present (Figure 2 and the Supporting Information). These results indicate the establishment of an immunological memory. In addition to IgG_1 and IgM antibodies, $IgG_{2a,b}$, IgG_3, and IgA antibodies were also induced to some extent. The difference in the isotype profiles compared to those of antisera induced

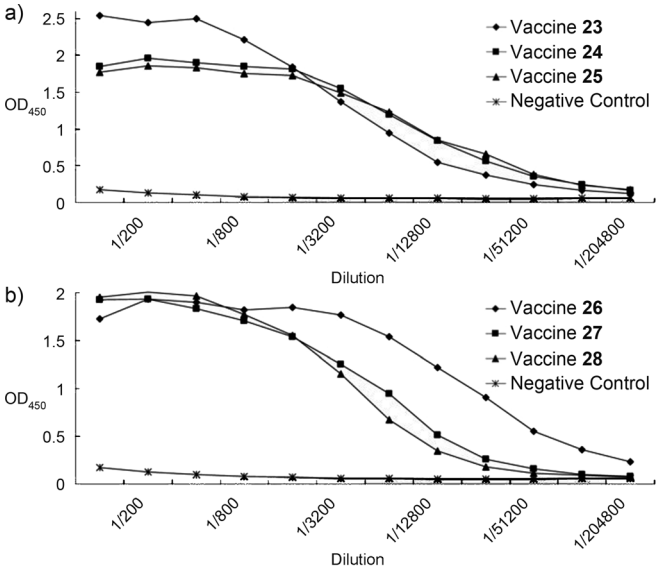

Figure 1. ELISA of the antisera induced by vaccines **23**–**25** (a) and **26**–**28** (b) after the third immunization; microtiter plates coated with **11**–**16**. Negative control used the sera of mice that had not been immunized.

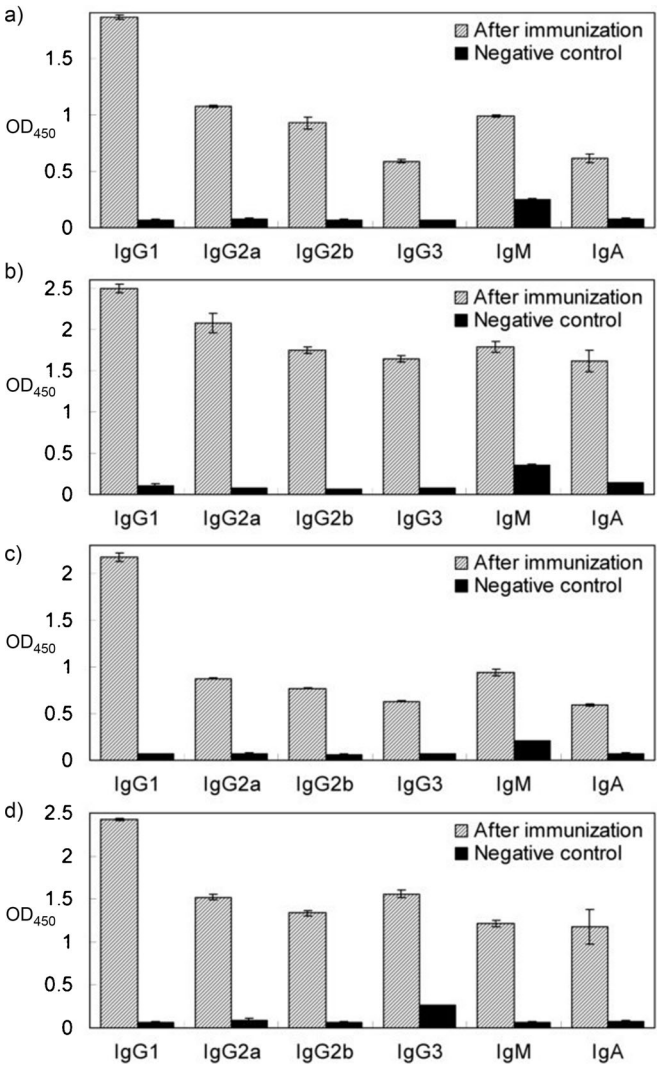

Figure 2. Determination of the isotypes of the antibodies induced by the vaccine **1** (a), **3** (b), **24** (c), and **27** (d) after the third immunization.

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by MUC1 glycopeptide tetanus toxoid vaccines,[2e,h] in particular the amounts of IgM and IgA antibodies, may be traced back to the BSA as the carrier protein.

The binding of antibodies induced by the synthetic vaccines **1**–**4** and **23**–**28** to MUC1 molecules expressed on human breast tumor cells MCF-7[2b] was determined by flow cytometry (FACS) analysis. The tumor cells were incubated with the induced sera diluted to 1:50. After washing, secondary rabbit antimouse antibodies carrying fluoresceinisothiocyanate as the fluorescent label were added to the cells. The cells recognized by the antibodies from the mouse antisera showed fluorescence and were counted by flow cytometry analysis. As shown in Figure 3, cells incubated with buffer solution (black) and those treated with sera of mice

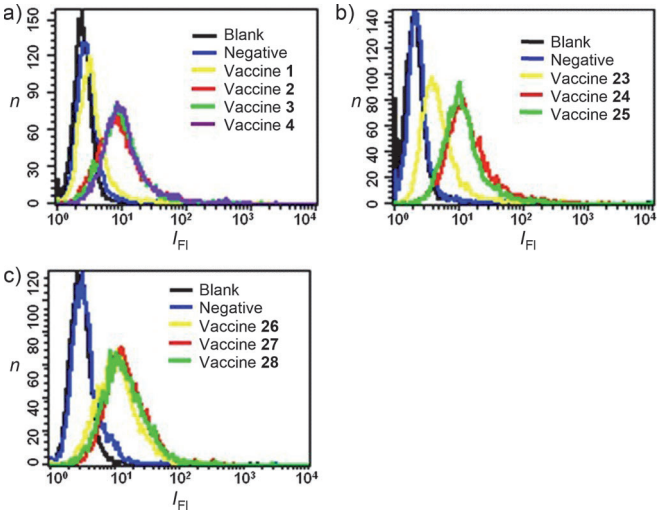

Figure 3. Analysis of the binding of the antisera to MCF-7 breast tumor cells: vaccines **1**–**4** (a), vaccines **23**–**25** (b), vaccines **26**–**28** (c). Blank: incubation of the cells with PBS buffer; Negative: incubation of the cells with the sera of mice that had not been immunized. I_{FI}: maximum fluorescence intensity measured during the passage of a cell through the measuring device; *n*: number of cells.

before immunization (blue) did not bind the fluorescent-labeled antimouse antibodies (negative control). The mouse serum induced by the non-glycosylated vaccine **1** had shown strong titers and predominant IgG-isotype antibodies; however, its binding to the tumor cells was very weak (Figure 3a, yellow line). The serum induced by vaccine **23** carrying the STn antigen at S15 also exhibited relatively weak binding to the tumor cells (Figure 3b, yellow line). This is surprising because a tetanus toxoid conjugate containing a closely related glycopeptide antigen induced antibodies that strongly recognized MCF-7 tumor cells. The antibodies in the sera induced by other MUC1 glycopeptide vaccines **24**–**28** showed strong binding to the tumor cells.

These results give evidence that strong immune responses can be induced in wild-type mice with tumor-associated MUC1 glycopeptide vaccines based on BSA as the carrier protein. Similar to previously reported results obtained with other vaccine constructs,[2e,g,k] these BSA-conjugated vaccines also induce IgG-isotype antibodies, which exhibit strong binding to the tumor-associated MUC1 glycoprotein expressed on MCF-7 tumor cells. It is of particular interest that the glycosylation at site T9 within the immunodominant PDTRP peptide epitope has a positive influence on the immunogenicity of the synthetic vaccines. According to these findings, BSA can replace the very expensive tetanus toxoid as the immune-stimulating carrier protein in exploratory immunization studies of synthetic MUC1 antitumor vaccines.

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