Organic & Biomolecular Chemistry

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View Article Online
DOI: 10.1039/C7OB02976D

Journal Name

ARTICLE

Synthetic and Immunological Studies on Trimeric MUC1 Immunodominant Motif Antigens-based Anti-cancer Vaccine Candidates

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Therapeutic vaccines have been regarded as a very promising treatment modality against cancer. Tumor-associated MUC1 is a promising antigen for the design of antitumor vaccines. However, body's immune tolerance and low immunogenicity of MUC1 glycopeptides limited their use as effective antigen epitope of therapeutic vaccines. To solve the problem, we chose the immune dominant region of MUC1 VNTRs. We designed and synthesized its linear trivalent glycopeptide fragments and coupled the fragments with BSA. Immunological evaluation indicated that the antibodies induced by glycosylated MUC1 based vaccine **11** had a stronger binding than non-glycosylated **10**. The novel constructed antigen epitopes have the potential to overcome the weak immunogenicity of natural MUC1 glycopeptides and deserve further research.

Introduction

Immunotherapy has become an important strategy to treat cancer, which is likely to avoid the side effects of traditional chemical therapy or radiotherapy. Cancer immunotherapy requires rational design of vaccine candidates that reflect phenotypic distinctions between normal and malignant cells.[1] Tumor-associated MUC1 is a promising antigen for the design of cancer vaccines. MUC1 is a transmembrane glycoprotein found on almost all types of epithelial tumor tissues. The extracellular domain of MUC1 consists of multiple variable number tandem repeats (VNTR) comprising 20 amino acids of the sequence HGVTSAPDTRPAPGSTAPPA, including 5 potential O-glycosylation sites.[2, 3] The types of carbohydrates are different between normal glycosylated MUC1 and aberrantly glycosylated MUC1. The common tumor associated carbohydrate antigens (TACAs) are Tn, Thomsen-Friedenreich (TF), sialyl Tn and sialyl TF.[4]

Numerous studies have been conducted to develop antitumor vaccines based on MUC1 glycoprotein.[5-9] There may be the following reasons for their insufficiency. One reason is that the immunogenicity of MUC1 antigen itself is very low and MUC1 is T cell-independent antigen. What is more, the body has immune tolerance to MUC1 as it is autoantigen. Targeted at these problems, scientists have done many exploratory studies. As to the issue of low immunogenicity of MUC1, researchers have conjugated MUC1 with different immunoenhancers such as proteins[10, 11], T epitopes[5, 12, 13] and adjuvants[6, 14-16]. For example, Kunz group has attached the $Pam_3CSKKKK$ lipopeptide to tumor-associated MUC1 glycopeptides to give fully synthetic vaccines;[14] Our group has fully synthesized MUC1-based antitumor vaccines with FSL-1 lipopeptide as the adjuvant.[15] To overcome the immune tolerance of MUC1, many groups have synthesized MUC1 analogues to improve its immunogenicity. For example, Ye group has synthesized some fluorine-containing STn antigens, and the vaccination results on mice showed that these modifications significantly improved the antigenicity;[17-19] Spadaro group has designed multiple units of immunodominant B-cell epitope PDTRP MUC1 core sequence as novel B epitope to constructvaccines.[20]

Finn group has reported that, compared with unglycosylated MUC1 VNTR, MUC1 VNTR containing TACAs have more potential to break self-tolerance in MUC1 transgenic mice. In their view, compared with unglycosylated MUC1 which is more self-like, the glycopeptide is a more foreign-like epitope.[21-23] Based on the previous reports, we presume that MUC1 glycopeptide antigens will induce stronger immune response compared with tumor associated carbohydrate antigens or peptide antigens.

In this paper, we chose the immune dominant region of MUC1 variable number of tandem repeats (VNTRs), namely PDTRPAP. We then designed and synthesized its linear trivalent glycopeptide fragments, and coupled the fragments with the carrier protein BSA (according to Fig.1, candidate 10 when R is H, and candidate 11 when R is Tn). The immunological evaluation of these vaccines is also reported. Through this design, we consider there are two advantages. First, the newly designed and synthesized glycopeptide fragments are non-natural, which may break the body's immune

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† Electronic Supplementary Information (ESI) available: HPLC analyses, NMR data and MS data of (glyco)peptides, MS data of (glyco)peptides and BSA conjugates.See DOI: 10.1039/x0xx00000x

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tolerance following the enhancement of the immunogenicity. Second, due to multivalent effect, the linear trivalent glycopeptides may have a stronger binding with B cell receptors, and then induce better immunogenicity.

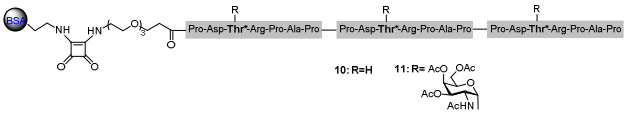
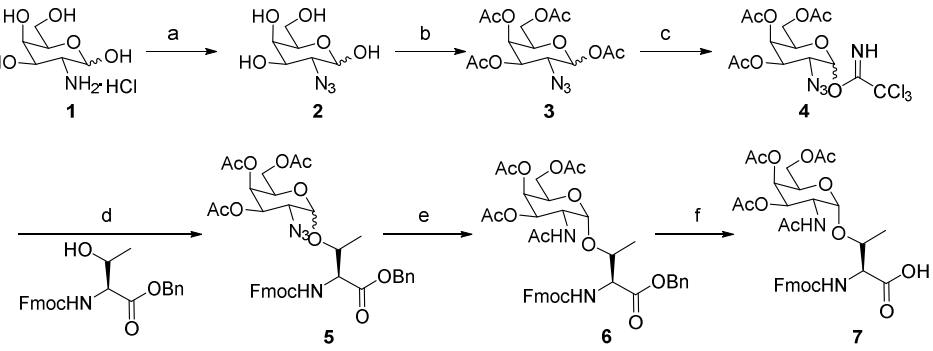


Fig.1 Structure of vaccine candidates with linear trivalent immune dominant region of MUC1 VNTR glycopeptide fragments conjugated to BSA.

Results and discussion

Before the preparation of vaccine candidates, we optimized the synthetic route of the Tn antigen (Scheme 1) in a large scale, as Tn is the most basic structural unit of preparing MUC1-based antitumor vaccines. To this end, we developed a synthetic route which is summarized in Scheme 1. As shown in Scheme 1, starting from D-galactosamine hydrochloride, we took advantage of imidazole-1-sulfonyl azide, an azide agent developed by our group previously [24], and synthesized 2-azidogalactose in large scale. After peracetylation and selective deacetylation on the C1 position, the intermediate was reacted with trichloroacetonitrile in the presence of DBU, and 2-deoxy-2-azidogalactoside donor **4** was afforded. **4** was then reacted with threonine with protecting groups through Schmidt glycosylation, forming the isomers 5 (α/β, 4:1). The α/β-anomers could be easily seperated by column chromatography after reduction and acetylation of **5**. After catalytic hydrogenation of **6**, protective Tn block **7** was obtained. To the best of our knowledge, it was the most safe preparation of this block at over 50 g level [25], which had the potency to be applied to large scale preparation in industry.

Preparation of vaccine candidates were carried out through the solid-phase peptide synthesis (SPPS) using 2-chlorotrityl resin preloaded with Fmoc-Proline.[26] The peptide synthesis was performed using Fmoc amino acids utilizing 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and N,N-diisopropylethylamine (DIPEA). Glycosylated Fmoc-amino acid was introduced by using the more reactive 1-[dis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt) and DIPEA. Ac_2O/Pyridine = 2:1 was chosen as the capping condition. 20% piperidine in DMF was chosen as the deprotection condition of the Fmoc group. Side chains were deprotected and the glycopeptide was detached from the resin with 90% TFA, 5% TIPS, and 5% H_2O. The *O*-acetyl groups on Tn antigen were removed under basic conditions using methanolic MeONa solution (pH=10~11), peptide **8** and glycopeptide **9** were isolated by semi-preparative RP-HPLC with the yield of 55% and 42% respectively. Treatment of the (glyco)peptides with diethyl squarate in aqueous solution (pH=8) and conjugation the obtained squaric acid monoamides with BSA in a buffer solution (pH=9.5)[27] afforded target vaccine candidates **10** and **11** after ultrafiltration with the membrane of 30 kDa (Scheme 2).



Scheme 1 Synthetic route of Tn antigen **7**

Reagents: (a) Imidazole-1-sulfonyl azide, Cu_2SO_4, K_2CO_3, MeOH (89%) α:β = 2:3; (b) Ac_2O, pyridine (92%) α:β = 3:7; (c) DMAPA, THF~~/MeOH~~; then CCl_3CN, DBU, CH_2Cl_2 (85% over two steps) α:βmixture, α>95%); (d) TMSOTf, 4Å MS, CH_2Cl_2 (76%) α:β=4:1; (e) AcSH, pyridine (70%); (f) Pd/C, H_2, EtOAc (94%).

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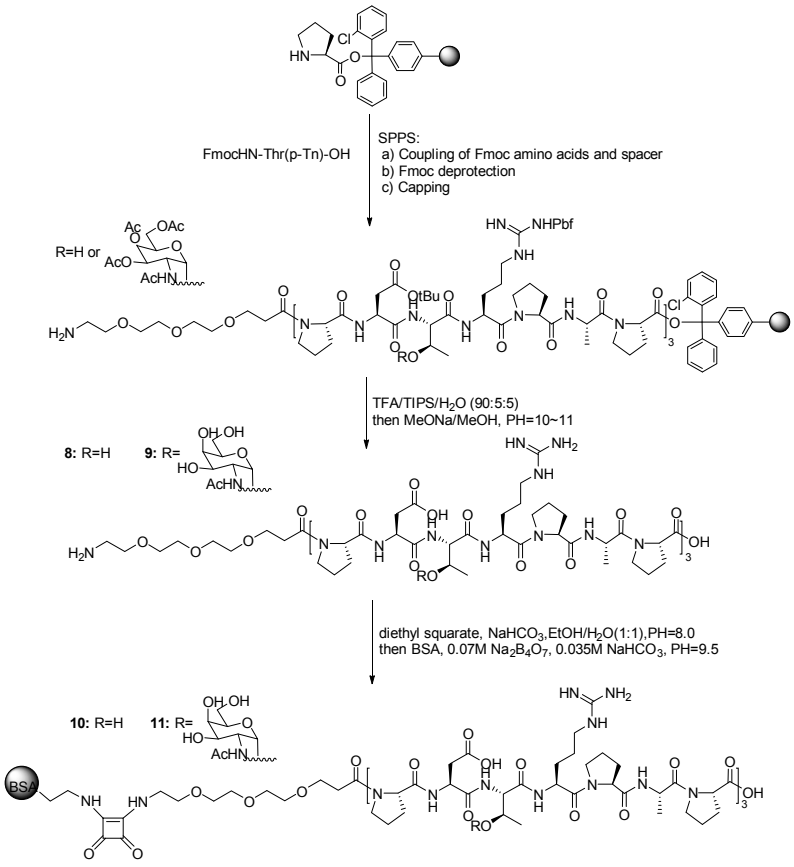

Scheme 2 Solid-phase synthesis of vaccine candidate **10** (R=H) and **11** (R=Tn)

To evaluate the immunogenicity of these vaccines, groups of female BALB/c mice (3 mice/group) were immunized biweekly four times via subcutaneous injection of vaccine candidate **10** and **11** (10 μg each time). Complete Freund's adjuvant (CFA) was used for the first immunization, incomplete Freund's adjuvant (IFA) for each subsequent booster. One week after last injection, the sera were collected for immunological evaluation (Fig.2). The antibody levels were analyzed by enzyme-linked immunosorbent assay (ELISA). The microtiter plates were coated with unconjugated (glyco)peptides **12** and **13** (see ESI) .

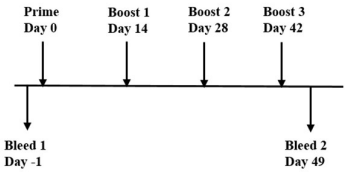

Fig.2 Immunization and bleed schedule

As shown in Fig.3, both unglycosylated vaccine candidate **10** and glycosylated vaccine candidate **11** elicited high levels of IgG antibodies. Antibody titer of vaccine candidate **10** was higher compared to glycosylated vaccine candidate **11**, which was in agreement with previous result.[28]

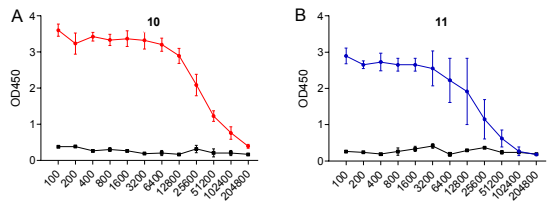

Fig.3 ELISA analysis of IgG to ~~of~~ the antisera induced by vaccine candidates **10** (A) and **11** (B). The microtiter plates for the evaluation of the sera induced by BSA-conjugated vaccines were coated with the peptide(**12**, MUC1-20) and glycopeptide(**13**, MUC1(Tn)-20) respectively. The sera collected before immunization were used as control. Data are shown as mean ± SD.

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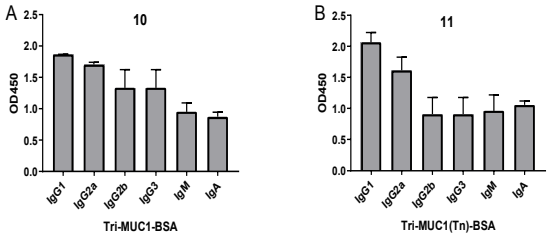

Fig.4 Isotype antibodies of IgG1, IgG2a, IgG2b, IgG3, IgM and IgA induced by vaccine candidates **10** (A) and **11** (B) were analyzed by ELISA. The microtiter plates for the evaluation of the sera induced by BSA-conjugated vaccines were coated with the peptide(**12**, MUC1-20) and glycopeptide(**13**, MUC1(Tn)-20) respectively. Data are shown as mean ± SD.

To further investigate the immune responses, the antibody isotypes elicited by vaccine candidates were tested by ELISA. IgG1, IgG2a, IgG2b, IgG3, IgM, IgA are included (Fig.4). According to the results of ELISA experiments, it's proved that all isotypes are high and higher IgG1 are elicited. On the basis of previous study, high levels of IgG1 signify Th2 responses [29] as IgG1 production is mainly activated by interleukin 4 (IL-4), a cytokine produced by type 2 T-helper (Th2) cell. High levels of IgG2a are reflection of Th1 responses. Therefore, the IgG2a/IgG1 ratio represents the relative contribution of the Th1/Th2 pathways to the immune response. [30] Vaccine candidate **10** and **11** elicited highest amount of IgG1, so they mainly activate Th2 cell, which is closely related to humoral immunity. Hence, the cluster effect of these glycopeptide vaccines resulted in a high contribution of Th2 response pathway during the immune reaction.

The specificity of the produced antibodies induced by the vaccine candidates binding to MCF-7 breast cancer cells were investigated by fluorescence-activated cell sorting (FACS) analysis. Both of the antisera induced by vaccine candidates **10** and **11** showed binding with human breast cancer cells. Compared with antisera from non-glycosylated vaccine **10**, glycosylated vaccine **11** showed stronger binding, though the titers of vaccine **10** are higher than vaccine **11** (Fig.5). This result might be due to the specificity of the antisera induced by vaccine **10** was poor compared with vaccine **11**.

Immunofluorescence assay was then performed to investigate the binding affinity of the antisera with cancer cells further. MCF-7 cells were used to conduct the experiment. As shown in Fig.6, both of the antisera induced by vaccine candidates **10** and **11** showed considerable binding with MCF-7 cells. Between them, the antisera of vaccine **11** showed stronger binding than vaccine **10**. The result was consistent with previous ELISA and FACS experiment.

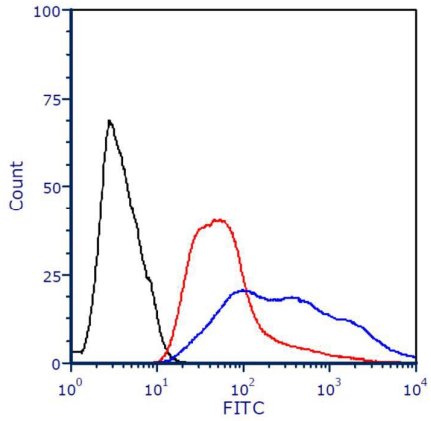

Fig.5 Flow cytometric analysis of the antisera binding with cancer cells. The binding of vaccine candidates **10** (red) and **11** (blue) immunized antisera with MCF-7. The sera from pre-immune mice were used as control (black).

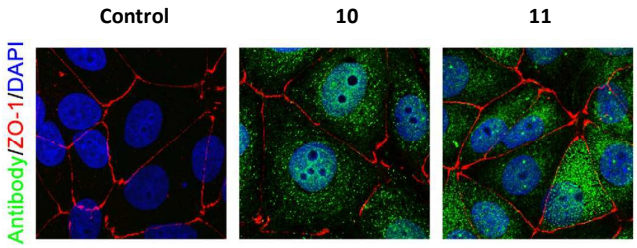

Fig.6 Immunofluorescence of MCF-7 cells. The cells were fixed, permeabilized and reacted with antisera (1:50 dilution) from different groups (normal group, anti-10 group, anti-11 group). DAPI staining was used to label cell nucleus (blue), ZO-1 staining was used to label cell membrane (red), FITC-labeled anti-mouse antibody was used to label the anti-sera.

Conclusions

Overall, in order to break the body's immune tolerance and overcome the low immunogenicity of MUC1 glycopeptides as effective antigen epitope of therapeutic vaccines, two linear trivalent glycopeptides of the immune dominant region of MUC1 VNTRs were designed and synthesized. In the preparation of these glycopeptides, we developed a novel synthetic route for large scale synthesis of Tn antigen through the use of safe imidazole-1-sulfonyl azide reagent. These glycopeptides were conjugated to carrier protein BSA to produce the vaccine candidates **10** and **11**. Immunological evaluation indicated that both of them induced considerable humoral immunity, and might mainly activate Th2 response pathway during the immune reaction. Compared with than non-glycosylated MUC1 based vaccine **10**, the antibodies induced by glycosylated **11** had a stronger binding through FACS analysis and immunofluorescence assay. According to these findings, the novel constructed antigen epitopes based on the immune dominant region of MUC1 VNTRs, especially the glycosylated one, have the potential to overcome the weak

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immunogenicity of natural MUC1 glycopeptides and deserve further research.

Experimental section

Chemical Synthesis

All reagents were purchased from commercial sources and were used without further purification unless otherwise noted. All solvents were available with commercially dehydrated or freshly dehydrated and distilled prior to use. Reactions were monitored using Thin Layer Chromatography (TLC) on silica gel GF254 plates with detection by short wave UV fluorescence (λ = 254 nm) after being stained with p-anisaldehyde solution (ethanol/panisaldehyde/acetic acid/sulfuric acid 135:5:4:1.5) or 10% phosphomolybdic acid in EtOH, followed by heating on a hot plate. Column chromatography was conducted by silica gel (200-300 mesh) with petroleum ether and ethyl acetate as eluent. ^{1}H and ^{13}CNMR spectra were obtained using a Bruker AV 400 MHz spectrometer at 400M Hz and 100M Hz respectively. Signals are reported in terms of their chemical shift (δ in ppm) relative to $CDCl_3$ (^{1}H, 7.26 and ^{13}C, 77.16), CD_3OD (^{1}H, 3.31 and ^{13}C, 49.00), $(CD_3)_2SO$ (^{1}H, 2.50 and ^{13}C, 39.52). Coupling constants are reported in hertz. High-resolution electrospray-ionization mass spectra (HRESIMS) were obtained on a Varian QFT-ESI mass spectrometer. Matrix-assisted laser desorption/ionizationtime of flight mass spectra (MALDI-TOF MS) were performed using 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxy cinnamic acid (CHCA) as matrix on Varian 7.0T FTMS instrument. Reversed-phase HPLC separations were performed on a Waters system 2487 using solution A (0.1% trifluoroacetic acid in 100% acetonitrile) and solution B (0.1% trifluoroacetic acid in 100% water) for elution. UV absorption signals were detected with an UV detector at a wavelength of 220nm.

2-azido-2-deoxy-D-galactose (**2**)

The compound was synthesized using our previously reported method. D-galactosamine hydrochloride (46.6 g, 216 mmol) and anhydrous potassium carbonate (44.8 g, 324 mmol) were dissolved in methnol (350 mL). $CuSO_4$ (568 mg, 3.20 mmol) was added and the mixture was stirred in dark for 0.5 h. Freshly prepared imidazole-1-sulfonyl azide[31] (240 mL, 180 mmol) was added and the mixture was stirred overnight. The mixture was filtered off through Celite and the solvent was concentrated in vacuo. The crude product was purified by flash chromatography (CH_2Cl_2: MeOH, 5:1) to give **2** as a light yellow solid (32.83 g, 89%, α:β = 2:3).

^{1}H NMR (400 MHz, CD_3OD) δ 5.18 (d, *J* = 3.5 Hz, 0.4H, H-1α), 4.43 (d, *J* = 7.1 Hz, 0.6H, H-1β), 4.04 – 3.94 (m, 1H), 3.90 (d, *J* = 2.8 Hz, 0.4H), 3.77 (m, 0.6H), 3.75 – 3.65 (m, 2H), 3.50 – 3.42 (m, 1H), 3.39 (m, 1H).

The spectral data were consistent with the reported results in the literature.[32]

3,4,6-tri-O-acetyl-2-azido-2-deoxy-D-galactopyranose (**3**)

The compound **2** (32.8 g, 160.05 mmol) was dissolved in pyridine (300 mL). Acetic anhydride (78 mL) was added dropwise in an ice-water bath under the protection of argon and the mixture was stirred overnight. The solution was diluted with dichloromethane and washed with 1M HCl, water and brine in turn. The organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography (petroleum ether/ethyl acetate, 5:1) to give **3** (65.92g, 92%, α:β = 3:7).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.27 (d, *J* = 3.6 Hz, 0.3H, H-1α), 5.52 (d, *J* = 8.4 Hz, 0.7H, H-1β), 5.43 (d, *J* = 2.8 Hz, 0.3H, H-4α), 5.33 (d, *J* = 3.2 Hz, 0.7H, H-4β), 5.27 (dd, *J* = 10.8, 2.8 Hz, 0.3H, H-3α), 4.87 (dd, *J* = 10.8, 3.2 Hz, 0.7H, H-3β), 4.23 (t, *J* = 6.8 Hz, 0.3H, H-5α), 4.13 – 4.01 (m, 2H), 3.99 (t, *J* = 6.4 Hz, 0.7H, H-5β), 3.90 (dd, *J* = 10.8, 3.6 Hz, 0.3H, H-2α), 3.79 (dd, *J* = 10.8, 8.4 Hz, 0.7H, H-2β), 2.15 (s, 2.1H), 2.13 (s, 0.9H), 2.12 (s, 3H), 2.03 (s, 0.9H), 2.02 (s, 2.1H), 1.99 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 170.0, 169.9, 169.8, 169.6, 168.7, 168.6, 92.8, 90.4, 71.7, 71.3, 68.7, 68.6, 66.9, 66.2, 61.1, 61.0, 59.7, 56.8, 20.9, 20.8, 20.6, 20.5, 20.5.

3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl trichloroacetimidate (4)

To a solution of **3**(65.91 g, 176.14 mmol) in THF/DCM (1:1, v/v, 150 mL) was added DMAPA (109.66 mL, 880.69 mmol). The mixture was stirred at room temperature for 2 hours. The solution was diluted with dichloromethane and washed with saturated ammonium chloride solution and brine. The organic layer was dried over Na_2SO_4 and concentrated. The afforded colourless oil was dissolved in dehydrated dichloromethane (1000 mL) in an ice-water bath. CCl_3CN (176.14 mL, 1.76 mol) and DBU (17.61mL, 140.91 mmol) were added successively under the protection of argon. The mixture was stirred for 45 min. The solution was diluted with dichloromethane and washed with saturated ammonium chloride solution and brine. The organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography (petroleum ether/ ethyl acetate, 1:1) to give **4** (71.24 g, 85% over two steps, α/β mixture, α>95%)

^{1}H NMR (400 MHz, $CDCl_3$, α) δ 8.79 (s, 1H), 6.50 (d, *J* = 3.6 Hz, 1H), 5.54 (d, *J* = 2.4 Hz, 1H), 5.37 (dd, *J* = 11.2, 3.2 Hz, 1H), 4.41 (t, *J* = 6.8 Hz, 1H), 4.14 (dd, *J* = 11.2, 6.8 Hz, 1H),4.07 – 4.01 (m, 2H), 2.17 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H).

The spectral data were consistent with the reported results in the literature.[33]

N-(9H-Fluorene-9-yl)-methoxycarbonyl-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine benzylester (**5**)

The isomers of **4** (71.22 g, 149.43 mmol), Fmoc-Thr-COOBn (58.11 g, 136.32 mmol), and 4 Å MS (2.0 g) in dehydrated CH_2Cl_2 (140 mL) was stirred at RT under an argon atmosphere for 1 h. After being cooled to -20℃, TMSOTf (6.99 mL, 38.62 mmol) was added, and the reaction was stirred at RT for another 2 h. When TLC showed that the reaction was completed, saturated aq. $NaHCO_3$ was added to quench the reaction, and it was then diluted with CH_2Cl_2. Molecular sieves were filtered through Celite. The solution was washed with saturated ammonium chloride solution and brine. The organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography (petroleum ether/ ethyl acetate, 6:1) to give **5** (84.59 g, 76%，α:β=4:1).

^{1}H NMR (400 MHz, $CDCl_3$, α) δ 7.77 (d, *J* = 7.4 Hz, 2H), 7.62 (d, *J* = 7.3 Hz, 2H), 7.35 (m, 9H), 5.68 (d, *J* = 9.2 Hz, 1H), 5.44 (br s, 1H), 5.31 – 5.16 (m, 4H), 4.91 (d, *J* = 3.3 Hz, 1H), 4.50 – 4.35 (m, 4H), 4.29 – 4.20 (m, 2H), 4.08 (d, *J* = 6.3 Hz, 2H), 3.59 (dd, *J* = 11.2, 3.6 Hz, 1H), 2.15 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H), 1.35 (d, *J* = 5.9 Hz, 3H).

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The spectral data were consistent with the reported results in the literature.[34]

N-(9H-Fluorene-9-yl)-methoxycarbonyl-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester(**6**)

Compound **5** (84.58 g, 114.43 mmol) was dissolved in thioacetic acid/ pyridine (2:1, v/v, 700 mL). The mixture was stirred at room temperature for 24 hours. The solution was diluted with dichloromethane and washed with saturated ammonium chloride solution and brine. The organic layer was dried over Na_2SO_4 and then concentrated. The crude product was purified by column chromatography (petroleum ether/ ethyl acetate, 3:2) to give **6** (60.71 g, 70%). (Notice: The crude product here was α/β isomers and could be separated easily by column chromatography)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.63 (d, *J* = 7.4 Hz, 2H), 7.51 – 7.22 (m, 9H), 5.99 (m, 1H), 5.87 (m, 1H), 5.38 (d, *J* = 2.3 Hz, 1H), 5.19 (d, *J* = 10.8 Hz, 1H), 5.11 – 5.07 (m, 2H), 4.80 (d, *J* = 3.6 Hz, 1H), 4.56 – 4.37 (m, 3H), 4.37 (m, 1H), 4.27 – 4.23 (m, 2H), 4.19 (m, 1H),4.08 (m, 2H), 2.16 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.30 (d, *J* = 6.3 Hz, 3H).

The spectral data were consistent with the reported results in the literature.[34]

N-(9H-Fluorene-9-yl)-methoxycarbonyl-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine (**7**)

Compound **6** (60.70 g, 80.12 mmol) was dissolved in ethyl acetate. 10% Pd–C (15 g) was added. The mixture was stirred under H_2 atmosphere for 3 hours. The catalyst was removed by filtration thoroughly. The combined filtrate was concentrated under vacuum and the residue was purified by column chromatography (DCM/MeOH, 6:1) to give **7** (50.4 g, 94%).

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.91 (d, *J* = 7.6 Hz, 2H), 7.74 (dd, *J* = 7.2, 3.1 Hz, 2H), 7.64 (d, *J* = 9.4 Hz, 1H), 7.57 (d, *J* = 9.8 Hz, 1H), 7.43 (m, 2H), 7.33 (m, 2H), 5.31 (d, *J* = 2.5 Hz, 1H), 5.05 (dd, *J* = 11.6, 3.2 Hz, 1H), 4.81 (d, *J* = 3.7 Hz, 1H), 4.45 – 4.39 (m, 2H), 4.30 (m, 2H), 4.23 (m, 2H), 4.15 (m, 1H), 4.03 (d, *J* = 6.0 Hz, 2H), 2.11 (s, 3H), 2.00 (s, 3H), 1.91 (s, 3H), 1.84 (s, 3H), 1.18 (d, *J* = 6.4 Hz, 3H).

The spectral data were consistent with the reported results in the literature.[34]

Immunological studies

All the animal experiments were conducted at Beijing Institute of Pharmacology and Toxicology, followed the protocol of the Institutional Animal Care and Use Committee, which was in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. Female Balb/c mice (6-8 weeks) were obtained and housed in institute's SPF grade animal facility, maintaining on a 12-hour light/dark cycle with free access to water and laboratory chow.

Mice were vaccinated via subcutaneous injections of 10 μg vaccine candidates in 100 μL PBS weekly for four times total. Complete Freund's adjuvant was used for the first immunization, incomplete Freund's adjuvant for each subsequent booster. Blood samples were collected via tail vein seven days after the last vaccination and the anti-sera were prepared for further analysis and validation.

Antibody titer analysis

The specific antigens, namely 20μg/mL (glyco)peptides **12** and **13**, respectively, were coated on the high-binding 96-well plates and blocked by 10% fetal bovine serum followed by incubating the gradient diluted corresponding antisera. The rabbit anti-mouse HRP-labeled IgG secondary antibody with 1/2000 dilution was then applied. TMB ELISA Substrates solution (Thermo, USA) was applied and 1M H_2SO_4 solution was added to stop the reaction before the plated being read at 450 nm by a plate reader.

Antibody Isotyping ELISA

For the qualitative determination of antigen specific antibody isotype in the antisera, the Mouse Monoclonal Antibody Isotyping kit (Sigma-Aldrich, USA) was applied here. The measurement was carried out according to the kit manual. The specific antigens, namely 20μg/mL (glyco)peptides **12** and **13**, respectively, were coated on the high-binding 96-well plates and blocked by 10% fetal bovine serum followed by incubating the diluted corresponding antisera (1:1000). Each of the isotype antibodies from the kit, including goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA with 1/2000 dilution, was then applied, followed by rabbit anti-goat HRP-labeled antibody. TMB ELISA Substrates solution (Thermo, USA) was applied and 1M H_2SO_4 solution was added to stop the reaction before the plated being read at 450 nm by a plate reader.

Binding between antigen specific antibody and MCF-7 cells

MCF-7 cells were cultured in DMEM culture medium containing fetal bovine serum (FBS, 10%) at 37°C. The cells were digested with 0.25% (w/v) trypsin solution and washed three times with PBS solution. The 5×10^5 cells suspensions were incubated with antiserum (1:50 in PBS) at 4°C for 1h. After washing with PBS three times, the cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody (diluted 1:1000 in PBS containing 1% FBS) at 4°C for 1h. After washing three times with PBS, the cells were suspended in washing buffer (1 mL) and filtered through 200-mesh sieve, FACS analysis was conducted on BD FACS Aria III flow cytometry.

Immunofluorescence studies

MUC1 expressing MCF-7 cells were cultured in 24-well plates. The cells were fixed with 4% paraformaldehyde buffer for 0.5 hour. The fixed cells were reacted with the antisera (1:50 dilution) from different groups at 4 °C overnight. For nuclear staining, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI). For cell membrane staining, the cells were stained with ZO-1. For immunofluorescence staining of the antibodies, the cells were incubated with fluorescein-isothiocyanate (FITC) labeled goat anti-mouse IgG (1:100 dilution) for 30 minutes. Photographs were acquired with a laser scanning confocal microscope (Leica, Wetzlar, Germany). All photographs were taken at the same magnification.

Conflicts of interest

The authors declare no competing financial interests.

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DOI: 10.1039/C7OB02976D

Acknowledgement

This work was supported by National Natural Science Foundation of China (21332006, 21572105), Natural Science Foundation of Tianjin (17JCZDJC32800) and the Fundamental Research Funds for the Central Universities.

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DOI: 10.1039/C7OB02976D

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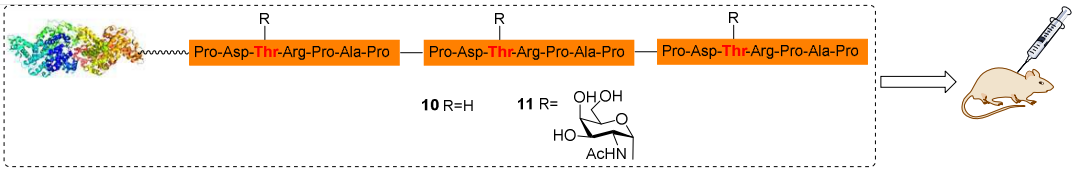

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