Supporting information

Design of a MUC1-based tricomponent vaccine adjuvanted with FSL-1 for cancer immunotherapy

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Materials and instruments

Matrix-assisted laser desorption/ionization time of flight mass spectra (MALDI-TOF MS) were performed using sinapinic acid (SA) or α-cyano-4-hydroxycinnamic acid (CHCA) as matrix on UltrafleXtreme MALDI-TOF/TOF (Brucker, Germany). Reversed-phase HPLC separations were performed on a Waters system 2487 using solution A (0.1% trifluoroacetic acid in acetonitrile) and solution B (0.1% trifluoroacetic acid in water) for elutions. UV absorption signals were detected with an UV detector at a wavelength of 220nm. Semi-preparative HPLC was used for separation and purification of the peptides on a C-18 column (10 µm, 10×250 mm, 100 µL per injection) at a flow rate of 2 mL/min. Mouse IgG antibody and Monoclonal Antibody Isotyping kit were purchased from Sigma-Aldrich (USA). ELISA kits detecting IL-6 and IL-12 were purchased from Biolegend (San Diego, CA, USA). MCF-7 and B16-MUC1 cell lines were purchased from China Infrastructure of Cell Line Resources.

General Procedure for peptides synthesis

(Glycolipo)peptides were synthesized as we previously reported. The solid-phase peptide synthesis (SPPS) for the preparation of vaccine candidate 1 was conducted using 2-chlorotrityl resin preloaded with Fmoc-Alanine (loading: 0.13 mmol g⁻¹, 3.85 g, 0.5 mmol). Fmoc amino acids were introduced with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 4.0 equiv), 1-hydroxybenzotriazole (HOBt, 4.0 equiv), N,N-diisopropylethylamine (DIPEA, 8.0 equiv). The introduction of glycosylated amino acid was conducted using more reactive 1-[dis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU, 2.0 equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 2.0 equiv), DIPEA (4.0 equiv) and its acetyl moieties were removed by MeONa/MeOH (pH = 10–11). Fmoc-Pam₂Cys-OH was coupled to the sequence with HATU/HOAt (2.0 equiv) and DIPEA (4.0 equiv). The protecting groups of side chain were removed and the glycopeptide was detached from the resin by adding 90% TFA, 5% TIPS, and 5% H₂O. The glycolipopeptide was purified by semi-preparative HPLC on a C18 column. Compound 2, 3, 4 and 5 were prepared in a similar way. The yield of HPLC is defined as the purification yield.

Analytical data for MUC1-(lipopeptide)
**Compound 1**

H-S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine-Gly-Asp-Pro-Lys-Pro-Lys-Ser-Phe-Gly-Gln-Tyr-Ile-Lys-Ala-
Asn-Ser-Phe-Ile-Gly-Ile-Thr-Val-Gly-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-
Thr-Ala-Pro-Pro-Ala-OH.

Analytical HPLC: Rt (retention time) = 21.9 min (50-75% of acetonitrile and 0.1% trifluoroacetic acid over 30 min
on a C-18 column, λ=220 nm). Yield 25% (10.8 mg).

ESI-MS: m/z for C_{250}H_{400}N_{60}O_{73}S [M+3H]^{3+} calcd 1815.31, found 1816.70; [M+4H]^{4+} calcd 1361.73, found 1362.35;
[M+5H]^{5+} calcd 1089.59, found 1090.30 ; [M+6H]^{6+} calcd 908.16, found 908.90.
MALDI-TOF-MS: C$_{250}$H$_{400}$N$_{60}$O$_{73}$S $^+ [\text{M+H}]^+$ calcd 5443.9226, found 5443.983.

**Compound 2**

H-S-[2,3-Bis[palmitoyloxy]-2RS-propyl]-(R)-cysteine-Gly-Asp-Pro-Lys-Pro-Lys-Ser-Phe-Gly-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Gly-His-Val-Thr-Ser-Ala-Pro-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-OH.

**Analytical HPLC:** Rt (retention time) = 16.1 min (50-75% of acetonitrile and 0.1% trifluoroacetic acid over 30 min on a C-18 column, $\lambda=220$ nm). Yield 29% (12.0 mg).
ESI-MS: m/z for C_{242}H_{387}N_{59}O_{68}S [M+3H]^3+ calcd 1747.62, found 1748.8; [M+4H]^4+ calcd 1310.97, found 1311.80; [M+5H]^5+ calcd 1048.97, found 1049.60; [M+6H]^6+ calcd 874.31, found 874.85; [M+7H]^7+ calcd 749.56, found 750.10.

MALDI-TOF-MS: C_{242}H_{387}N_{59}O_{68}S [M+H]^+ calcd 5240.8432, found 5241.212.
Compound 3

H-S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine-Gly-Asp-Pro-Lys-Pro-Lys-Ser-Phe-Val-Gly-Cys-His-Val-Thr-Ser-Ala-Pro-Asp-Arg-Pro-Ala-Pro-Gly-Thr-Ala-Pro-Pro-Ala-OH.

The compound was characterized in our previous report.

Compound 4

H-S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine-Gly-Asp-Pro-Lys-Pro-Lys-Ser-Phe

Analytical HPLC: Rt (retention time) = 6.603 min (10-100% of acetonitrile and 0.1% trifluoroacetic acid over 10 min on a C-18 column, λ=220 nm). Yield 49% (9.6 mg).
ESI-MS: m/z for C_{84}H_{141}N_{15}O_{17}S [M+2H]^{2+} calc 833.0, found 833.7; [M+3H]^{3+} calc 555.7, found 556.2.

The compound was characterized in previous report\(^2\).

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\text{GHGVTSAPDTRPAPGSTAPPA} \\
\text{QYIKANSKFIGITEGHGVTSAPDTRPAPGSTAPPA}
\]

**Compound 5**

H-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Asp-Glu-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Thr-Ala-Pro-Pro-Ala-OH.

Analytical HPLC: Rt (retention time) = 9.3 min (16-40% of acetonitrile and 0.1% trifluoroacetic acid over 30 min on a C-18 column, \(\lambda=220\) nm). Yield 38% (10.1 mg).
ESI-MS: m/z for C_{164}H_{259}N_{45}O_{55} [M+3H]^{3+} calcd 1247.3, found 1248.0; [M+4H]^{4+} calcd 935.7, found 936.3.

MALDI-TOF-MS: C_{164}H_{259}N_{45}O_{55} [M+H]^+ calcd 3739.8926, found 3740.081
H NMR (400 MHz, D2O) δ 8.47 (s, 1H), 7.23 – 7.11 (m, 5H), 7.08 (d, J = 6.9 Hz, 2H), 6.96 (d, J = 8.0 Hz, 2H), 6.66 (d, J = 8.2 Hz, 2H), 4.57 – 4.53 (m, 3H), 4.52 (d, J = 6.9 Hz, 2H), 4.48 (d, J = 8.8 Hz, 2H), 4.45 – 4.41 (m, 4H), 4.40 – 4.36 (m, 3H), 4.32 – 4.29 (m, 2H), 4.27 (s, 3H), 4.25 – 4.19 (m, 8H), 4.16 – 4.08 (m, 7H), 4.00 – 3.94 (m, 4H), 3.92 – 3.86 (m, 3H), 3.85 – 3.81 (m, 5H), 3.74 (d, J = 5.0 Hz, 4H), 3.71 – 3.64 (m, 7H), 3.59 (s, 3H), 3.53 – 3.43 (m, 8H), 3.16 (s, 1H), 3.08 – 3.02 (m, 3H), 2.98 – 2.93 (m, 1H), 2.89 – 2.81 (m, 6H), 2.81 – 2.76 (m, 3H), 2.74 (d, J = 6.3 Hz, 1H), 2.67 (dd, J = 15.6, 6.5 Hz, 2H), 2.31 (d, J = 7.0 Hz, 2H), 2.26 – 2.20 (m, 4H), 2.18 – 2.07 (m, 6H), 2.00 – 1.95 (m, 4H), 1.92 – 1.84 (m, 14H), 1.79 – 1.72 (m, 6H), 1.59 – 1.51 (m, 8H), 1.49 – 1.45 (m, 2H), 1.31 – 1.24 (m, 5H), 1.24 – 1.18 (m, 15H), 1.11 – 1.07 (m, 3H), 1.07 – 1.02 (m, 10H), 0.82 – 0.75 (m, 10H), 0.72 – 0.66 (m, 16H).

Selected signals δ 8.47 (1H, Arg); 7.23 – 6.66 (11 H, Tyr, Phe, His-aryl group).
$^{13}$C NMR (101 MHz, D$_2$O, selected signals for carbonyl groups, Arg, Tyr and C-1) δ 176.84, 176.69, 176.46, 174.90, 174.88, 174.58, 174.29, 174.25, 174.19, 174.02, 173.80, 173.75, 173.73, 173.51, 173.34, 173.32, 173.27, 173.18, 173.16, 172.70, 172.64, 172.42, 172.20, 172.07, 171.97, 171.87, 171.64, 171.57, 171.52, 171.52, 171.46, 171.13, 171.09, 171.02, 171.00, 170.92, 170.80, 168.94, 168.72, 163.10, 162.74, 156.70 (Arg), 154.46 (Tyr), 98.48 (C-1).

Antigen for ELISA plate coating

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\text{NH}_2(CH_2CH_2O)_3CH_2CO-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(\alpha-D-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-OH}
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The compound was characterized in our previous report\(^1\).
FACS analysis of the antisera binding with B16-MUC1 cells

Fig. S1 FACS analysis of the antisera binding with B16-MUC1 cells. The sera from mice injected with PBS was used as control (black)

References
