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Design, Synthesis, and Preliminary Immunological Studies of MUC1-Based Antitumor Vaccines Adjuvanted with *R*- and *S*-FSL-1

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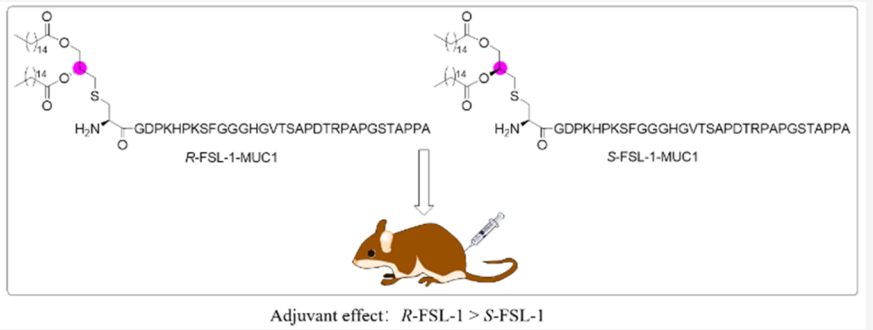

ABSTRACT: Fibroblast stimulating lipopeptide 1 (FSL-1) is the ligand of TLR2 and TLR6 and can be used as the vaccine adjuvant to prepare antitumor vaccines. However, FSL-1 is a stereoisomeric mixture that contains the *R* stereoisomer and *S* stereoisomer, and it is still unclear which stereoisomer has better adjuvant activities. In this work, we designed and synthesized MUC1-based antitumor vaccines adjuvanted with the stereoisomers *R*-FSL-1 and *S*-FSL-1, which were synthesized from the stereoisomeric building blocks *R*-Fmoc-Pam$_2$Cys-OH and *S*-Fmoc-Pam$_2$Cys-OH, respectively. Immunological evaluation indicated that both *R*-FSL-1 and *S*-FSL-1 can be used as adjuvants for the construction of MUC1-based antitumor vaccines, with *R*-FSL-1 showing a better adjuvant effect than *S*-FSL-1.

KEYWORDS: *FSL-1, MUC1, antitumor vaccines, immunological evaluation*

Vaccines have been widely used to prevent or treat cancer and infectious diseases by stimulating immune responses.[1,2] Carbohydrates and peptides with defined chemical structure have been employed as powerful antigens.[3,4] However, the immunogenicity of most antigens is very low, limiting their applications from bench to clinic.[5] To enhance the immunogenicity, a variety of vaccine carriers and adjuvants have been developed, such as carrier proteins,[6,7] nanoparticles,[8] polymers,[9] virus carriers,[10] toll-like receptor (TLR) ligands,[11] natural killer T (NKT) cell ligands,[12] and stimulator of interferon gene (STING) agonists.[13]

Fibroblast stimulating lipopeptide 1 (FSL-1, Pam$_2$-CGDPK HPKSF) is recognized by TLR2 and TLR6.[14−16] Our group first proved that FSL-1 can be used as a powerful vaccine adjuvant for the construction of antitumor vaccines.[17] Research by the Chiang group suggested that FSL-1 could be also used as an efficient adjuvant for intranasal EV71-vaccine immunization.[18] However, FSL-1 is a stereoisomeric mixture containing the *R* stereoisomer and *S* stereoisomer. Previous reports indicated that the chirality of the central carbon in the diacylglycerol group plays a very important role in TLR recognition.[19,20] Thus, it is of great interest to know the relationship between the structure and adjuvant activities of *R*-FSL-1 and *S*-FSL-1.

In this work, we compared the adjuvant effects of the *R* and *S* stereoisomers of FSL-1 in inducing tumor-antigen-specific antibodies and cellular immune response. Mucin 1 (MUC1), a glycoprotein that is overexpressed on the surface of tumor cells compared with normal cells, is one of the most commonly used tumor-associated antigens.[21−23] The extracellular domain of MUC1 is composed of multiple variable-number tandem repeats (VNTRs). Each VNTR contains 20 amino acids of the sequence HGVTSAPDTRPAPGSTAPPA, which has five potential O-glycosylation sites.[24] A variety of tumor vaccines based on MUC1 antigen have been reported,[25−32] and MUC1 was defined as the second prioritized cancer antigen for translational research by the National Cancer Institute.[33] Here

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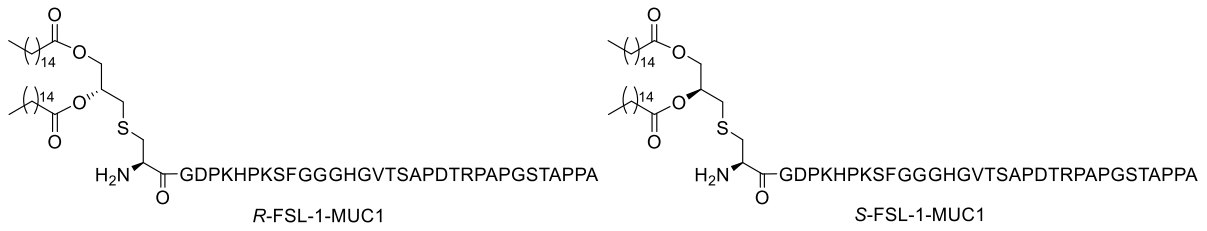
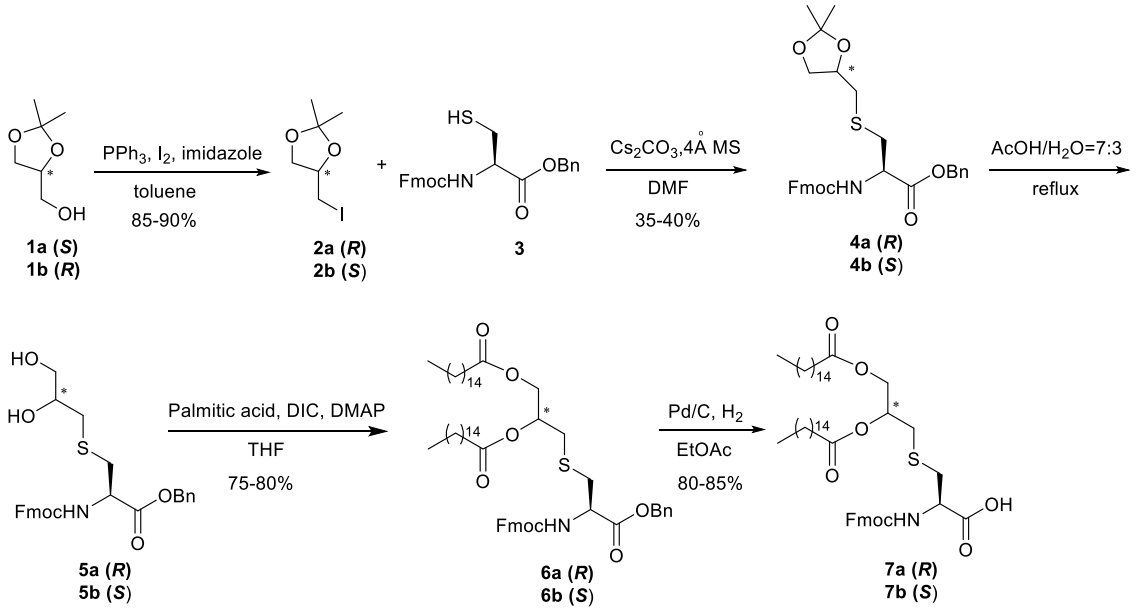


Figure 1. Structures of *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1.

Scheme 1. Synthesis of *R*-Fmoc-Pam$_2$Cys-OH (7a) and *S*-Fmoc-Pam$_2$Cys-OH (7b)



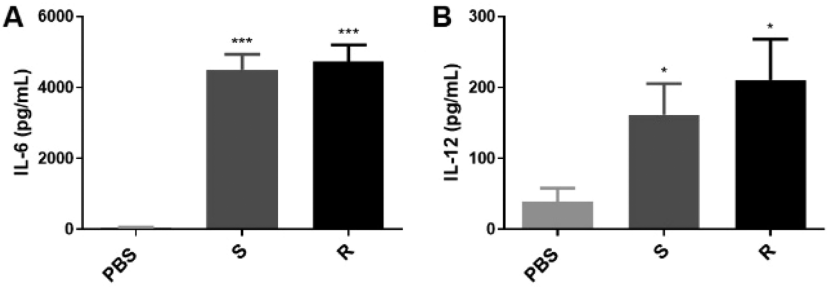


Figure 2. Effects of *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 on the production of pro-inflammatory cytokines in vitro. Shown are the amounts of (A) IL-6 and (B) IL-12 released in the culture supernatants of mouse peritoneal macrophages incubated with *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1. Data are shown as mean ± SD. Compared with the PBS group, *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

we used the unglycosylated MUC1 VNTR as the model antigen to construct vaccines, and *R*- and *S*-FSL-1 were covalently conjugated with MUC1 peptide (Figure 1).

As shown in Scheme 1, to prepare the vaccine candidates, we began with the synthesis of the building blocks *R*-Fmoc-Pam$_2$Cys-OH (**7a**) and *S*-Fmoc-Pam$_2$Cys-OH (**7b**). The respective starting materials (*S*)-(+)- and (*R*)-(−)-2,2-dimethyl-1,3-dioxolan-4-ylmethanol (**1a** and **1b**) were treated with triphenylphosphine, imidazole, and iodine in toluene to give **2a** and **2b** in yields of 85% and 90%, respectively. **2a** and **2b** were treated with compound **3** in the presence of cesium carbonate in anhydrous DMF under an argon atmosphere to give **4a** and **4b** in 35% and 40% yield, respectively.[17] Then **4a** and **4b** were deprotected using 70% acetic acid (AcOH/H_2O = 7:3) to give **5a** and **5b**. Palmitic acid was subsequently added to **5a** and **5b** with diisopropylcarbodiimide (DIC) as the dehydrant to give **6a** and **6b** in 75% and 80% yield, respectively. Finally, the hydrogenation of **6a** and **6b** catalyzed by Pd/C in EtOAc under 4 atm H_2 gave **7a** and **7b** in yields of 80% and 85%, respectively.

After **7a** and **7b** were in hand, we performed solid-phase peptide synthesis (SPPS) to prepare the vaccine candidates. The synthesis was carried out using 2-chlorotrityl resin preloaded with Fmoc-alanine. A Gly-Gly-Gly oligopeptide spacer was used to link MUC1 peptide and FSL-1 lipopeptide. Fmoc amino acids were introduced using HBTU/HOBt, while **7a** and **7b** was conducted by more reactive HATU/HOAt. Then the side-chain protecting groups were removed, and the lipopeptides were released from the resin by the addition of 95% TFA, 2.5% TIPS, and 2.5% H_2O (Scheme S1). The

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resulting crude lipopeptides were purified by reversed-phase HPLC to get the target lipopeptides.

To analyze the effects of *R*-FSL-1 and *S*-FSL-1 on the production of pro-inflammatory cytokines in vitro, we added the vaccine candidates to cultures of peritoneal macrophages harvested from C57BL/6 mice. The secreted pro-inflammatory cytokines (IL-6 and IL-12) were evaluated by ELISA (Figure 2). Both *R*-FSL-1 and *S*-FSL-1 induced the secretion of IL-6 and IL-12, with *R*-FSL-1 showing greater potential to induce the secretion of these cytokines.

To further evaluate the immune efficacies of the *R*-FSL-1- and *S*- FSL-1-based vaccine candidates, in vivo immunological experiments were then performed. Groups of female C57BL/6 mice were immunized on days 0, 14, 28, and 42 via intraperitoneal injection with *R*-FSL-1-MUC1, *S*-FSL-1-MUC1, or PBS solution. The sera were collected 1 week later, and the immune responses were then evaluated.

The IgG antibody titers were assessed first. As shown in Figure 3, both *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 elicited considerable IgG titers. There was no obvious difference between *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1, which means that they may have considerable potential to induce antibody production.

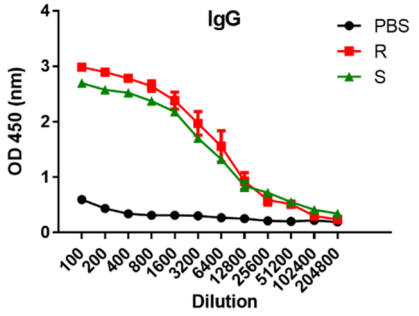

Figure 3. IgG antibody titers of the antisera induced by *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 vaccine candidates. Data are shown as mean ± SD.

The antibody isotypes were then analyzed by ELISA. The results showed that both *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 elicited high levels IgG1, IgG2b, IgG2c, and IgG3 antibody isotypes (Figure 4). The IgG1 isotype is related to Th2 immune response, and the IgG2c isotype is related to Th1 immune response. As shown in Figure 4, the main antibody isotype was IgG1 rather than IgG2c. The IgG1/IgG2c ratio is related to the correlation of Th2/Th1 immune responses, with ratios of >1 indicating a Th2 immune response. The results show that both *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 mainly induced Th2 immune response.

The binding affinities of the antisera induced by the vaccine candidates with tumor cells were then evaluated by fluorescence-activated cell sorting (FACS). MCF-7 human breast cancer cells, which express MUC1, were incubated with the antisera elicited by the vaccine candidates. Then fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody was added, followed by FACS detection. As shown in Figure 5, the antisera induced by both *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 showed significant binding activity with MCF-7 cells in comparison with the PBS group. Moreover, the antisera induced by *R*-FSL-1-MUC1 presented better binding capacity than that induced by *S*-FSL-1-MUC1.

To further evaluate the immune responses induced by the vaccine candidates, splenocytes of the mice immunized with the vaccine candidates were collected 1 week after the last immunization. After the splenocytes were cultured with MUC1 peptide (Figure S3), the cytokines of IL-4, IL-6, IL-12, and IFN-γ were tested. IL-4 and IL-6 are Th2-type cytokines, which are associated with the promotion of antibodies and humoral immune response. IFN-γ and IL-12 are Th1-type cytokines, which activate cell-mediated immunity and phag-

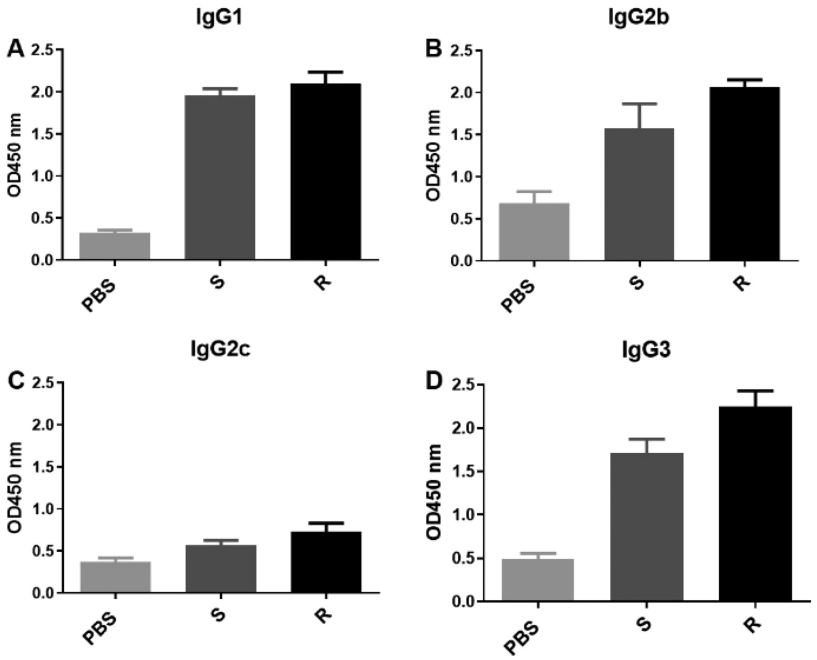

Figure 4. Analysis of the antibody isotypes (A) IgG1, (B) IgG2b, (C) IgG2c, and (D) IgG3 of the antisera induced by *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 vaccine candidates. Data are shown as mean ± SD.

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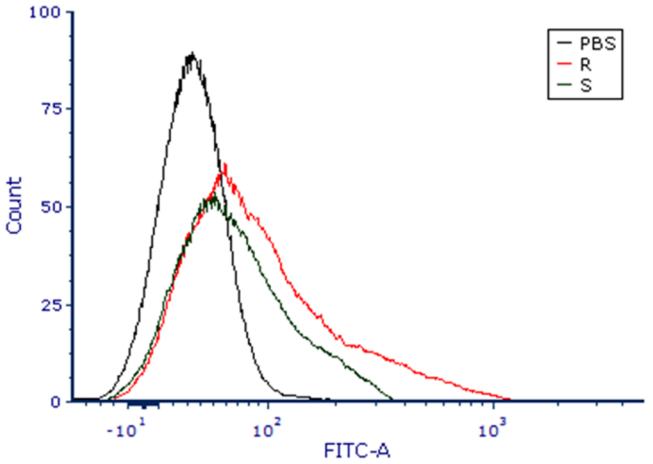

Figure 5. FACS analysis of the antisera elicited by *R*-FSL-1-MUC1 (red) and *S*-FSL-1-MUC1 (green) vaccine candidates. The PBS group (black) was used as a negative control.

ocyte-dependent inflammation. As shown in Figure 6, both *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 induced higher levels of IL-4, IL-6, IL-12, and IFN-γ than the PBS group. What is more, *R*-FSL-1-MUC1 induced higher levels of all four cytokines tested than *S*-FSL-1-MUC1.

On the whole, the vaccine adjuvant FSL-1 is a stereoisomeric mixture that contains the *R* stereoisomer and *S* stereoisomer. Vaccine candidates *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 were designed and constructed to compare the potential adjuvant effects of *R*-FSL-1 and *S*-FSL-1. As analyzed by the secretion of pro-inflammatory cytokines by peritoneal macrophages, *R*-FSL-1 exhibited better stimulatory capacity than *S*-FSL-1. Moreover, *R*-FSL-1-MUC1 stimulated higher antibody titers and cytokines than *S*-FSL-1-MUC1. As FSL-1 is the ligand of TLR2 and TLR6, the different immunocompetencies of *R*-FSL-1 and *S*-FSL-1 might be attributed to their binding affinities with TLR2 and TLR6, which needs to be further verified. Overall, the results indicate that *R*-FSL-1 could induce a stronger adjuvant effect than *S*-FSL-1.

In summary, the building blocks *R*-Fmoc-Pam$_2$Cys-OH (**7a**) and *S*-Fmoc-Pam$_2$Cys-OH (**7b**) for the solid-phase synthesis of *R*-FSL-1 and *S*-FSL-1 were synthesized in five steps in overall yields of 17% and 24%, respectively. From them we constructed *R*-FSL-1- and *S*-FSL-1-based vaccines with MUC1 peptide as the antigen. Cytokine profiles in vitro indicated that *R*-FSL-1 can promote peritoneal macrophages to secrete more pro-inflammatory cytokines of IL-6 and IL-12 than *S*-FSL-1. Immunological results indicated that *R*-FSL-1-MUC1 had more potency to induce antibodies, IgG isotypes, and anti-MUC1 binding affinity with MCF-7 cells than *S*-FSL-1-MUC1. Moreover, both *R*-FSL-1-MUC1 and *S*-FSL-1-MUC1 induced high levels of IL-4, IL-6, IL-12, and IFN-γ. To sum up, the work here indicates that both *R*-FSL-1 and *S*-FSL-1 can be used as adjuvants for the construction of vaccines, with *R*-FSL-1 showing a better adjuvant effect than *S*-FSL-1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00579.

Experimental details for chemical synthesis; ^{1}H and ^{13}C NMR, HPLC, and MS data and spectra; and immunological evaluation methods (PDF)

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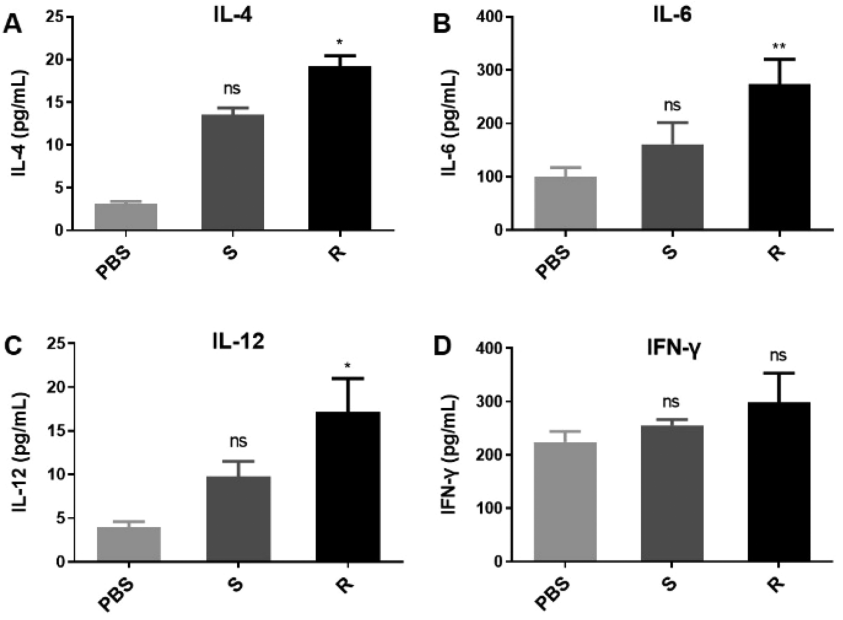

Figure 6. (A) IL-4, (B) IL-6, (C) IL-12, and (D) IFN-γ released in the culture supernatants of splenocytes isolated from immunized mice stimulated with MUC1 peptide (10 μg/mL). Data are shown as mean ± SD. Compared with the PBS group, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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Author Contributions

[†]Y.L. and B.Y. contributed equally. The manuscript was written through contributions of all authors. All of the authors approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TLR, toll-like receptor; NKT, natural killer T cell; STING, stimulator of interferon gene; FSL-1, fibroblast stimulating lipopeptide 1; MUC1, mucin 1; VNTR, variable-number tandem repeats; TACA, tumor-associated carbohydrate antigen; SPPS, solid-phase peptide synthesis; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting

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