Structure-based Design of Anti-cancer Vaccines: The Significance of Antigen Presentation to Boost the Immune Response

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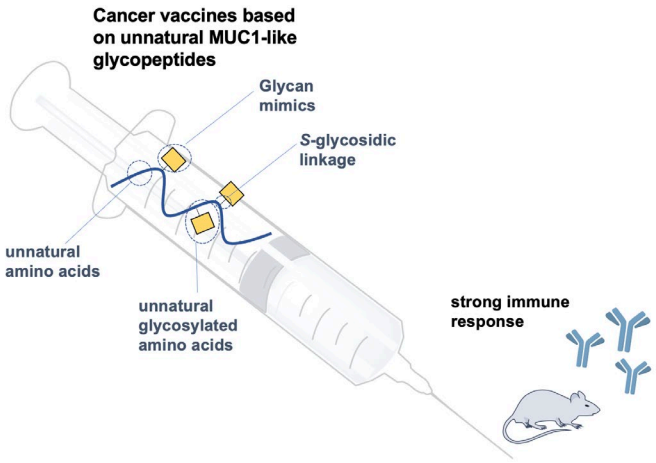
ABSTRACT

Immunotherapy, alone or in combination with other therapies, is being widely used against cancer. Glycoprotein Mucin 1 (MUC1), which is overexpressed and aberrantly glycosylated in tumor cells, is one of the most promising candidates to engineer new cancer vaccines. Within this context, the development of stable antigens that can boost a robust immune response is mandatory. We herein show the design and the biological evaluation in vivo of three vaccine candidates based on MUC1 glycopeptides that comprise unnatural elements in their structure. Precisely, by placing the Tn antigen (GalNAcα-O-Ser/Thr) in the design center, the chemical modifications cover changes at peptide backbone, glycosidic linkage, or carbohydrate level. Significantly, the three vaccines elicit a robust immune response in mice and produce antibodies that can be recognized by several human cancer cells. A relationship between the conformational alterations that the new elements prompt in the antigen presentation and the immune response obtained in mice has been established in all cases. According to our data, the development of efficacious vaccines based on MUC1 should engage surrogates that mimic the conformational space of aberrantly glycosylated MUC1 glycopeptides found in tumors.

GRAPHICAL ABSTRACT



KEYWORDS

cancer vaccine, immunotherapy, mucin, glycopeptide structure, non-natural antigens, tumor-associated carbohydrates, Tn antigen

INTRODUCTION

Vaccines have been one of the great discoveries of humanity that have made it possible to prevent many infections by pathogens and eradicate associated diseases.[1] While most of these vaccines are preventive and protect us against potential future invaders, therapeutic vaccines are intended to stop the development of a disease.[2] These latter ones are attracting considerable attention as potential weapons to fight cancer, as they are envisioned to boost the immune system to precisely eliminate tumoral cells without affecting the healthy tissues.[3] In this regard, it is well-established that some malignant cells express 'tumor-associated antigens' that can be recognized by the immune system.[4,5] In general, cancer vaccines are composed of a structurally defined antigen combined with an adjuvant. These antigens can be peptides, tumor-associated carbohydrate antigens (TACA), glycopeptides, glycolipids, or nucleic acids that encode a specific protein or peptide.[6] Within this context, glycoprotein MUC1 is one of the most promising targets as an effective antigen.[7] The extracellular domain of this protein displays the tandem-repeat (TR) sequence HGVTSAPDTRPAPGSTAPPA, in which the five threonine and serine residues are, in general, glycosylated via α-*O*-glycosidic linkages.[8] In healthy cells, the peptide backbone of this protein is attached to complex carbohydrates with diverse structures, being the first sugar an *N*-acetylgalactosamine (GalNAc) with further carbohydrate elongation.[9] On the contrary, in cells of various types of cancer, MUC1 is overexpressed and displays rather simple carbohydrates.[10,11] In these cases, the carbohydrate moiety is reduced to a GalNAc unit or relative short *O*-glycans. Consequently, tumor-associated MUC1 bears different TACAs, such as the Tn (GalNAcα-*O*-Ser/Thr)[12] or the sTn ((Neu5Acα2-6GalNAcα-*O*-Ser/Thr) determinants.[13] Also, the peptide sequence APDTRP has been demonstrated to be one of the most immunogenic fragments of the TR sequence, where glycosylation of the threonine residue with short glycans enhances the immune response.[14] In short, MUC1 is a good target for cancer immunotherapy as those glycan alterations and unmasked peptide can be recognized by the immune system while the risk of undesired effects can be minimized, as its expression in normal cells is residual.[15] However, it is important to note that mucin-like glycopeptides are poorly immunogenic, being unable to elicit a potent and long-lasting immune response.[16] To address this issue, these derivatives are conjugated to T-cell epitopes, protein carriers, nanoparticles or lipopeptides ligands. These components are essential immunostimulants and some of them allow for the presentation of many copies of the antigens. As a result, a plethora of multicomponent vaccines have been developed and evaluated in animal models[17–24] as well as in humans.[25]

However, the current vaccine prototypes had limited success and, therefore, further potential strategies are under development, including longer subunits, antigens combination, optimization of adjuvants, and combination with other therapies such as blockage of immune system tolerance factors.[26] A promising approach to overcome roadblocks is the use of 'artificial' antigens. These surrogates exhibit, in general, an improved chemical stability and bioavailability relative to natural ones, and keep a robust immune response.[27–30] Notably, most of the modifications accomplished to date are related to vaccines based on a single TACA antigen and not on a MUC1 glycopeptide. In these cases, the modifications involve the aglyconic part,[31–36] the glycosidic linkage, affording to *C*- or *S*-glycoside analogues,[37,38] or the carbohydrate, replacing specific hydrogen atoms by fluorine or chlorine atoms.[39,40] In the context of MUC1, a few vaccines based on glycopeptides that carry fluorinated T antigens (Galβ1-3GalNAcα-*O*-Ser/Thr) have proved to elicit a strong immune response in mice.[41,42]

The introduction of chemical modifications in natural glycopeptides may alter the conformational space of the novel antigens and, consequently, its presentation and the immune response produced by the designed vaccines. Because of this, we present herein structural studies, based on X-ray data, NMR experiments and molecular dynamics, on three MUC1-glycopeptides that feature unnatural Tn antigens. These customized antigens, recently used by us to formulate new vaccines, involve changes at the peptide backbone or modifications at the glycosidic linkage or carbohydrate moiety, as is shown in Figure 1. Our data provide valuable information at the atomic level to explain the impact these modifications could play in the immune response evoked by the new vaccines in mice.

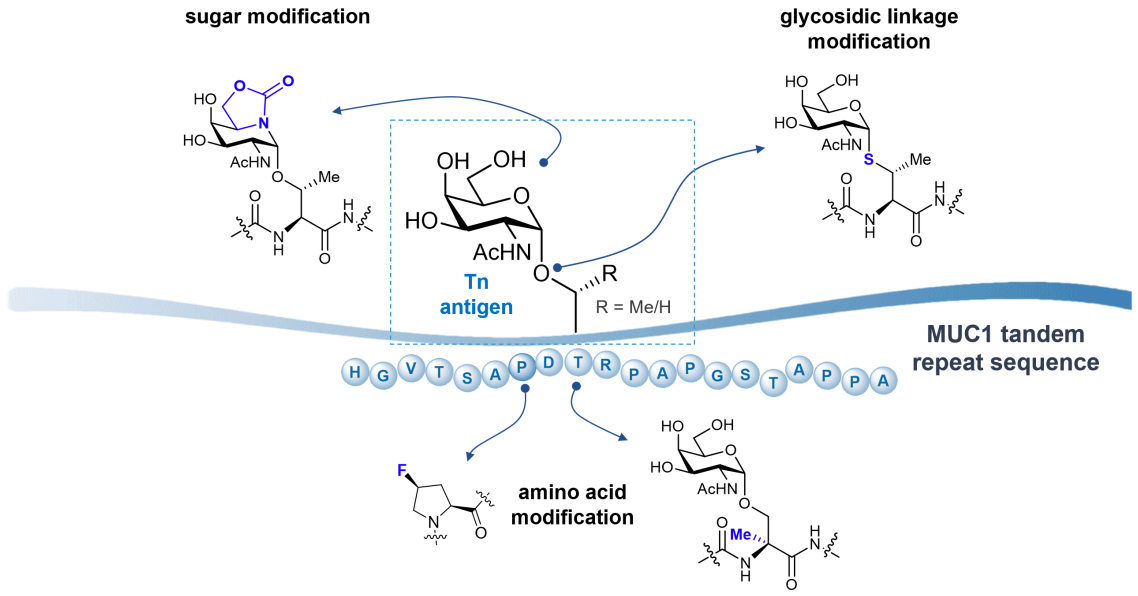


Figure 1. Chemically engineered cancer vaccines based on MUC1 developed by our group. Among other components, the new vaccines comprise the tandem repeat sequence of MUC1 in which an unnatural Tn antigen is incorporated.

RESULTS AND DISCUSSION

In the quest for an effective and safe vaccine, chemists are pursuing to control the activity of a promising vaccine by adjusting the structure and altering, in this way, the conformational properties of the designed antigens. This study aims to overview and deepen how these small chemical changes can impact the immune activity of three vaccines developed recently by our group.

1. Conformational differences between the two Tn antigens

As most of cancer vaccines based on MUC1 are bearing in their structure the Tn antigen (GalNAcα-*O*-Ser/Thr), we consider relevant to study the conformational preferences of these molecules and determine the differences (if any) between them. In general, the Tn antigen is referred to as a GalNAc unit linked via an α-*O*-glycosidic linkage to a serine or a threonine residue (**Tn-Ser** and **Tn-Thr**, respectively, Figure 2A), without specifying which of the two amino acids the sugar is linked. However, our group has studied the differences, in terms of conformational behavior and sugar presentation, between these two natural antigens in aqueous solution. The conformational analysis has been performed by NMR experiments (NOEs and coupling constants) which have been interpreted with molecular dynamics simulations.[43,44]

The analysis shows that **Tn-Thr** (Figure 2A) is rigid in solution, displaying the so-called 'eclipsed conformation' for the glycosidic linkage, with values of φ and ψ ≈ 80° and 120°, respectively.[44] The side chain, which is defined by χ^1 torsional angle, is also rigid and takes values around 60°. On the contrary, the **Tn-Ser** (Figure 2A) presents the exo-anomeric/*syn* conformation for the glycosidic linkage with φ and ψ values ~80° and ~180°, respectively.[43] The side chain of the serine residue is more flexible and presents the three possible staggered conformers. This dissimilar behavior is translated into a different orientation of the peptide backbone relative to the sugar moiety. In **Tn-Thr**, the carbohydrate lies almost perpendicular to the peptide but in **Tn-Ser** the GalNAc adopts a parallel orientation. Moreover, according to our structural studies, the water molecules in the first hydration shell of these determinant are distributed in a different way. Thus, while the **Tn-Thr** displays a water pocket that connects the NH groups of the GalNAc and threonine moieties (Figure 2B), in **Tn-Ser** the bridging-water molecule is localized between the NH of the

sugar and the carbonyl group of the glycosylated serine. These water pockets could be, together with direct sugar-peptide hydrogen bonds,[45] responsible for the extended conformation displayed by the peptide in solution in both compounds and the distinct behavior of the glycosidic linkages. Interestingly, the computational models have been experimentally validated by the crystal structure of an anti-MUC1 antibody (named SM3) in complex with different glycopeptides that comprise fluorinated **Tn-Thr** antigens (Figure 2C).[46] The presence of *N*-fluoroacetyl groups in the sugar increases the donor character of the NH group of GalNAc and allows to trap these localized water-bridging molecules, which confirms the results of the water pocket for the **Tn-Thr** case.

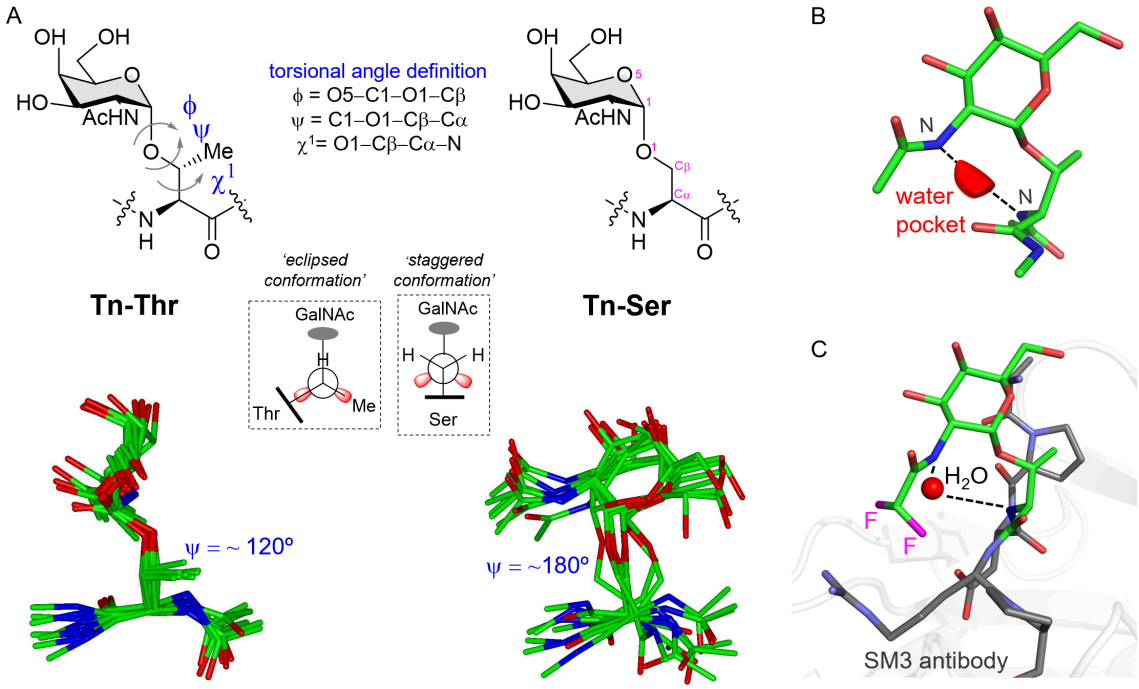


Figure 2. (A) Structural ensembles in solution[43,44] for the Tn antigen with a Thr (**Tn-Thr**) or a Ser (**Tn-Ser**) amino acid. The values of the torsional angle ψ of the glycosidic linkage, together with the definition of the torsional angles, are also shown. A Newman projection for the Cβ–O1 bond is also given, showing the staggered (**Tn-Ser**) and eclipsed (**Tn-Thr**) conformations. (B) Water-bridging molecule between the peptide fragment and the sugar for the **Tn-Thr** antigen derived from experiment-guided MD simulations.[44] (C) Views of the binding sites of the complex between a Tn-glycopeptide and the scFv-SM3 antibody (PDB ID: 6FZQ), showing the key water molecule between the *N*-difluoroacetyl group of the sugar and the amino group of the threonine residue.[46] The carbon atoms of the Tn-antigen are shown in green. The rest of the carbon atoms of the glycopeptide are in grey. Hydrogen atoms have been removed for clarity. The antibody is shown as a white cartoon.

To corroborate the active role of water molecules in the conformational properties of these two determinants, we have recently performed a thorough conformational analysis of them in the gas phase using infrared ion-dip analysis.[46] These studies indicate that the conformational space sampled by these derivatives is highly similar in absence of water to that presented by the **Tn-Ser** in water. Therefore, we propose that water in the first hydration shell forces the rotation around the glycosidic linkage in the threonine derivative to an 'eclipsed conformation', which shields the hydrophobic methyl group and allows optimal solvation of the polar region of the antigen.

It is important to note that these structural features are also displayed when the Tn antigens are incorporated into peptides. Figure 3A shows the most immunogenic fragment of MUC1 comprising the **Tn-Thr**.[47] The solution NMR-derived structure of this glycopeptide in water exhibits mainly an extended conformation for the peptide backbone and a rigid glycosidic linkage and side chain, which are identical to those found for the **Tn-Thr** solution.

2. Design and biological evaluation of cancer vaccines based on unnatural MUC1 glycopeptides

A plausible cause for the failures of the MUC1-based vaccines could be related to the sensitivity of the antigens to endogenous glycosidases, which reduces their bioavailability.[35] As aforementioned, an attractive strategy to enhance the effectiveness of cancer vaccines may be the use non-natural antigens.

2.1. The use of a Tn antigen comprising the non-natural amino acid α-methylserine

On this basis, we designed a Tn antigen variant that comprises the quaternary amino acid α-methylserine (MeSer)[48–50] and was integrated in the most immunogenic fragment of MUC1 (Figures 1 and 3B-left panel). From a structural viewpoint, and in agreement with the NMR/MD analysis, the glycosidic linkage adopts the geometry observed in the **Tn-Ser** analogue.[51] The methyl group at carbon-α forces the unnatural amino acid to display φ and ψ angles similar to those of a helix-like conformation in water, which is expected for α,α-disubstituted amino acids,[52] and makes the rest of the peptide and the side chain markedly flexible (Figure 3A).

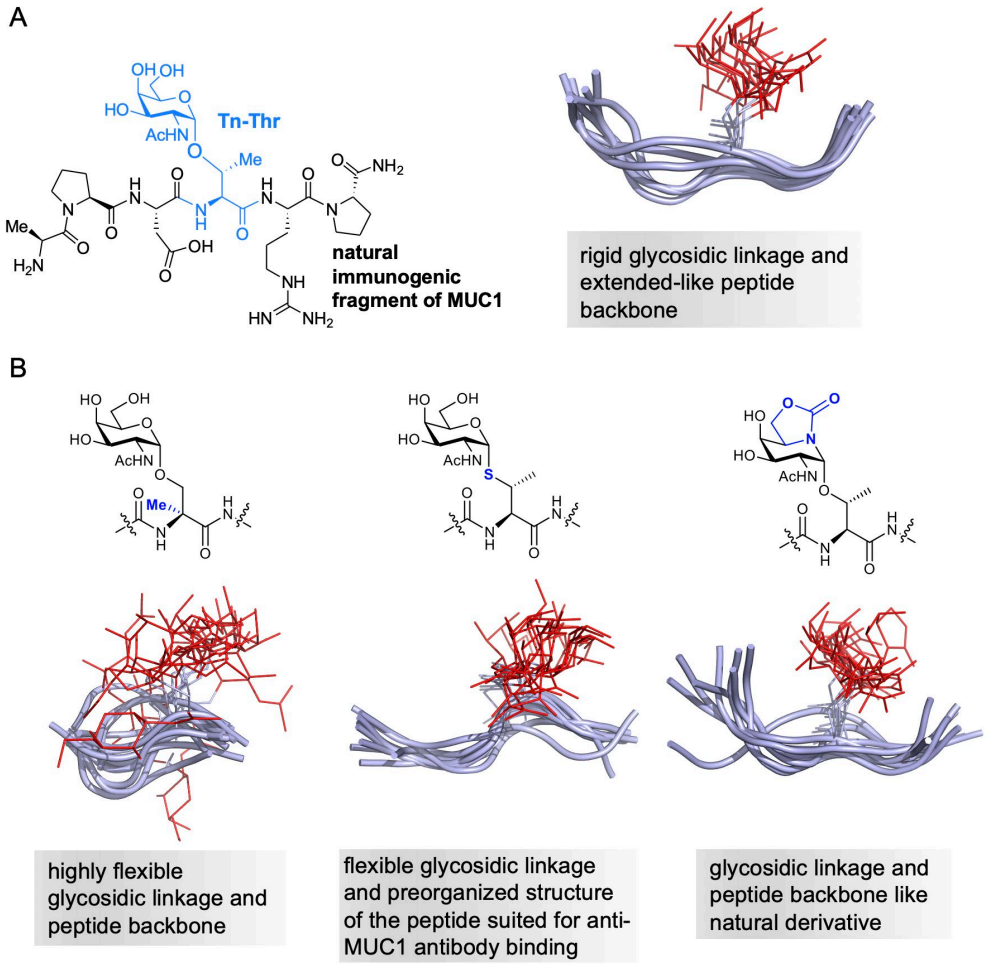


Figure 3. (A) Chemical structure of the most immunogenic fragment of MUC1, together with the structural ensembles derived from experiment-guided MD simulations. (B) Ensembles obtained from experiment-guided MD simulations for various unnatural glycopeptides comprising the most immunogenic fragment of MUC1 and Tn mimetics. The most relevant structural features for each derivative are also shown in a text box. The peptide backbone is represented as purple ribbons and the GalNAc unit as red sticks. Hydrogen atoms have been removed for clarity.

To study whether this conformational alteration could have any effect on the efficacy of an anti-cancer vaccine, we included the unnatural Tn antigen into the most immunogenic domain of a MUC1 sequence and designed a three-component vaccine,[51] similar to that previously reported by Boons and co-workers.[53] We demonstrated first that the unnatural glycosylated epitope has better stability in human serum related to the natural variant. As illustrated in Figure 4A, the vaccine consists of the unnatural MUC1 epitope covalently linked to a T-helper peptide. This fragment is also connected to a TLR2 agonist, whose aliphatic chains promote the formation of liposomes in solution (Figure 4B). These liposomes allow for the presentation of the antigens in a multivalent manner and, at the same time, can improve the half-life of the vaccine in mice. As shown in Figure 4C, the engineered vaccine elicited a potent immune response in mice, producing antibodies (mainly of subtype IgG1) that can recognize native MUC1 present on cancer cells (Figure 4D). Yet, in terms of vaccine design, it is important to note that the effectiveness of this unnatural antigen does not enhance the immune response relative to the natural analog with threonine. We conclude that the extra flexibility of the unnatural antigen could have a negative impact on interaction with the elements of the immune system, mitigating the production of antibodies.

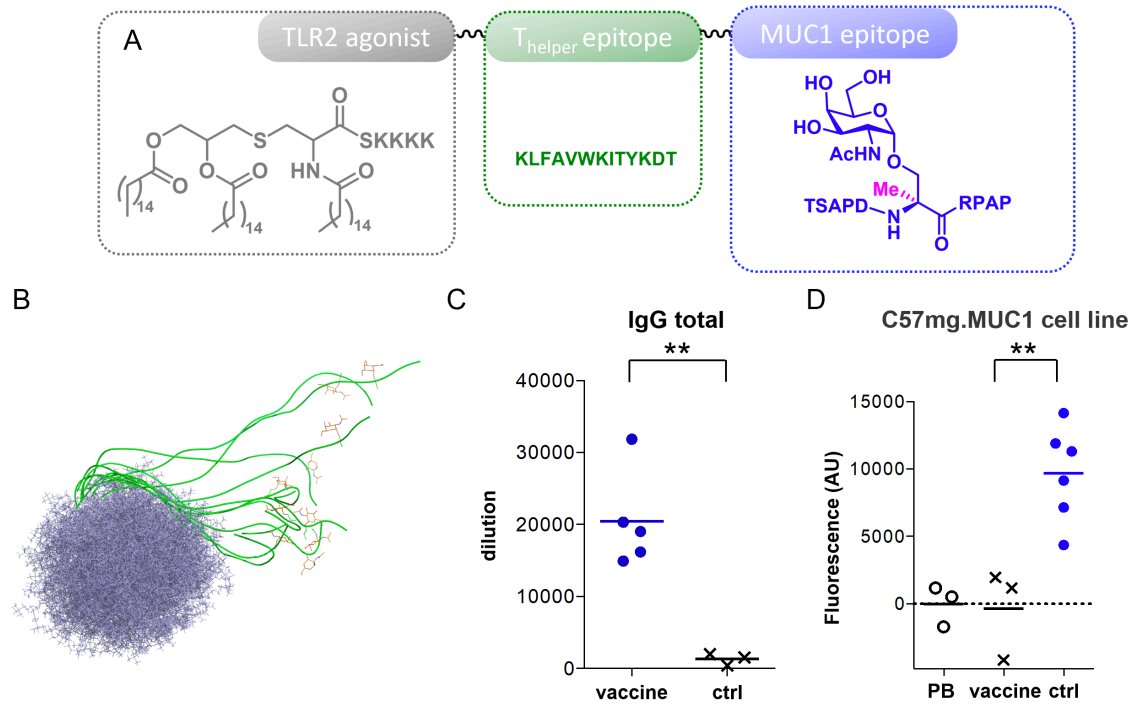


Figure 4. (A) Chemical structure of an anti-cancer vaccine incorporating the unnatural amino acid α-methylserine at the MUC1 epitope. This formulation is equivalent to that previously reported by Boons and co-workers using the natural MUC1 epitope. (B) Structural ensembles obtained from MD simulations on 65 copies of the vaccine. The peptide backbone is shown in green, the GalNAc unit in read and the liposomes in purple. (C) Anti-MUC1 antibody titers after 3 immunizations with either empty liposomes (n=3, negative control –ctrl–) or the vaccine in liposomes (n=5). ELISA plates were coated with BSA-maleimide-CTSAPDT(α-D-GalNAc)RPAP conjugate and titers were determined by linear regression analysis, plotting dilution *vs* absorbance. (D) Serum samples (1:50 diluted) were incubated with C57mg.MUC1 cells. These cells express tumor associated MUC1 on their surface. After incubation with FITC-labeled anti-mouse IgG antibody, the fluorescence intensity was assessed in cell lysates. AU indicates arbitrary fluorescence units. PB stands for pre-bleed. Each data point represents the titer for an individual mouse and the horizontal lines indicate the mean for the group of mice. Asterisks indicate statistically significant difference (**P< 0.01).

2.2. The use of an unnatural amino acid in the APDTRP region and a Tn antigen that displays an *S*-glycosidic linkage

Next, we performed a structure-guided design of a new generation of potent antigens in terms of affinity to anti-MUC1 antibodies. For this end, we proposed two approaches: the first one pursues the optimization, in solution, of the main structure recognized by the antibodies; the other one entails the improvement of antigen-antibody interactions. In the first approach, we did chemical modifications at the glycosidic linkage level. In this regard, an exciting observation about the structural features of MUC1 peptides is that α-*O*-glycosylation with GalNAc shifts the conformation of the underlying peptide into an extended structure in solution (Figure 3A).[45,47] This can be explaining by the occurrence of stabilizing interactions, such as hydrogen bonds or water-bridging molecules, between the peptide and the carbohydrate moiety (Figure 2B). However, the X-ray structure of the glycopeptide APDT*RP bound to an anti-MUC1 antibody (SM3) revealed a folded conformation around the glycosylated Thr.[54] In this case, the sugar shifts the structure of the peptide in solution away from that adopted upon antibody binding. This conformational entropic penalty can be counteracted by enthalpic contributions between the sugar moiety and the antibody, which results in a modest net increase in binding affinity (around 3-fold) for the glycosylated related to the unglycosylated peptide. Therefore, we proposed a rational approach based on single-atom substitution (O → S/Se) at the glycosidic linkage to obtain potent antigens with an improved affinity toward anti-MUC1 antibodies.[47] Taking into account that sulfur and selenium atoms are larger than oxygen, this simple modification increases the distance between the sugar and the peptide fragment and prevents the formation of stabilizing contacts between them. As a result, a helix-like conformation of the glycosylated residue is the most populated in solution (Figures 1 and 3B-middle panel). In turn, the glycosidic linkage becomes slightly more flexible due to the attenuation of the *exo*-anomeric effect caused by these large chalcogens atoms and adopts a main conformation in water as that recognized by the SM3 antibody. To our delight, the new glycopeptides comprising these mimetics of Tn determinants act as potent antigens, showing an improved affinity towards anti-MUC1 antibodies relative to the natural glycopeptide comprising the natural **Tn-Thr**.
In a parallel research line, we have observed that the X-ray structure of SM3 antibody bound to the peptidic antigen A**P**DTRP reveals that the Pro close to the *N*-terminal region (shown in bold letter) is engaged in CH/π interactions with the aromatic units of two tryptophan and a tyrosine of the antibody.[54] As demonstrated, this stabilizing interaction can be improved by increasing the polarization of the interacting C–H groups.[55–57] Towards that end, we added highly electronegative fluorine atoms to specific positions of the proline residue. The simple replacement of this proline by (4*S*)-4-fluoro-L-proline led to a significant increase of the staking interactions between these entities, which was experimentally corroborated by the analysis of the X-ray structure of the glycopeptide featuring this fluoroproline and the SM3 antibody.[58] The distance between the 5-membered ring of the Pro and the aromatic ring of the engaged tryptophan residue of the SM3 antibody was significantly shorter related to the native antigens previously reported by us and others.[54,59] This optimization of the complex antigen-antibody resulted in a high affinity of the engineered antigen towards the SM3 antibody.
Leading on from these results, we designed an unnatural glycopeptide (Figure 5A), which includes the complete tandem repeat sequence of MUC1 and features a thiothreonine glycosylated with GalNAc (***S*Thr***) and a (4*S*)-4-fluoro-L-proline (**fPro**).[47] Moreover, recent studies indicate that gold nanoparticles (AuNPs) can be used as efficient antigen carriers to establish humoral immunity against the tumor-associated form of MUC1 in mice.[60] Therefore, we conjugated this MUC1 derivate to AuNPs and a standard immunization strategy, similar to that employed in the previous study (section 2.1), was followed to test *in vivo* the immunogenic potential of the engineered potential vaccine. In this occasion, no external adjuvants were employed, demonstrating that the AuNPs *per se* can act as a suitable adjuvant. The analysis of the sera of the mice showed that the new vaccine can elicit a significant IgG antibody response, producing largely IgG1 antibodies (Figure 5B).

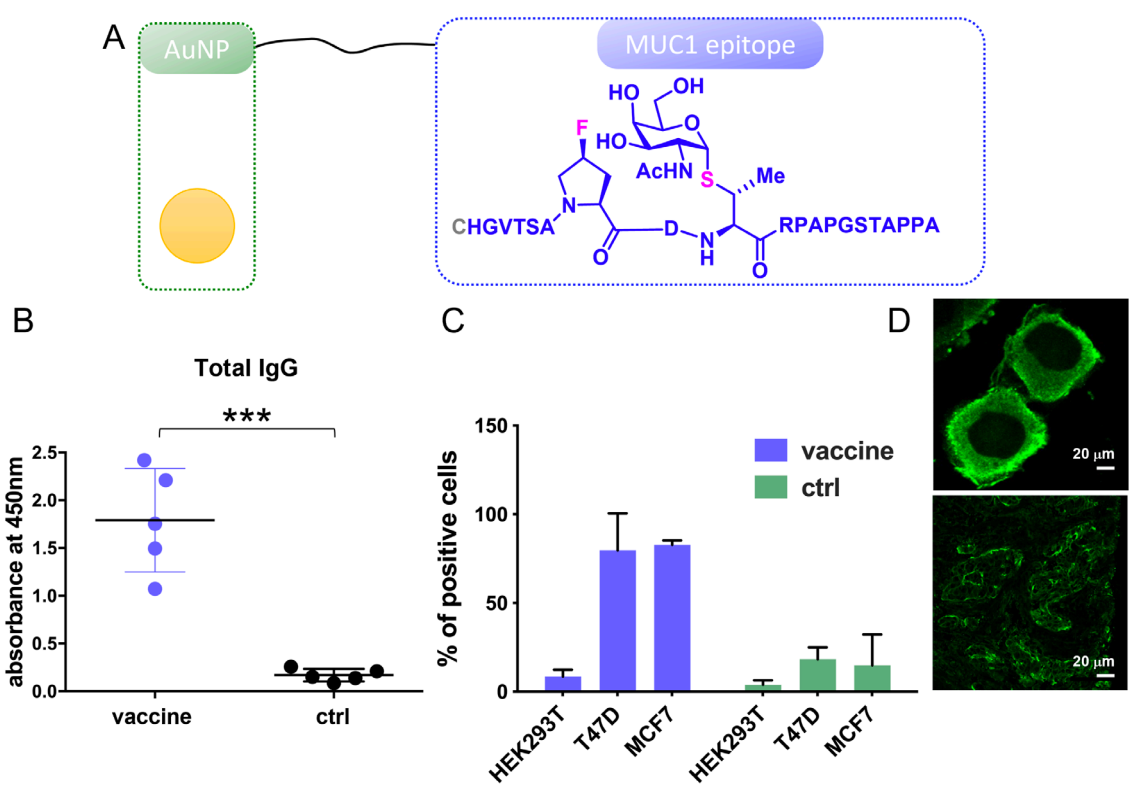


Figure 5. (A) Schematic representation of the anti-cancer vaccine based on a MUC1-glycopeptide featuring the thiothreonine glycosylated with GalNAc and a (4*S*)-4-fluoro-L-proline attached to gold nanoparticles. (B) Total IgG anti-MUC1 antibodies after immunizing mice (n = 5) with the vaccine and control (ctrl) group. The ELISA plates were coated with the glycopeptide used to formulate the vaccine but conjugated to bovine serum albumin. Horizontal lines indicate the mean for the group of five mice. Asterisks indicate statistically significant differences (***P < 0.005). A phosphate-buffered saline (PBS) solution was used as a negative control –ctrl–. (C) Staining of living cells with the antisera of mice immunized with the vaccine analyzed by flow cytometry. While MCF7 and T47D cell lines express tumor associated MUC1 on their surface, HEK293T do not displays this molecule on its membrane. (D) Upper panel: Confocal microscopy image shows that mice antisera after vaccination with the vaccine stain breast cancer cells T47D expressing tumor associated MUC1. Lower panel: The antisera of mice vaccinated with the unnatural vaccine positively stain tissue biopsies from breast cancer patients. Green = secondary anti-mouse IgG Alexa 488.

We also confirmed that the elicited antibodies can recognize selectively native tumor associated MUC1 antigens on human cancer cells. For this purpose, two human cancer cell lines that express tumor-associated MUC1 on their surface (MCF-7, T47D) and the human embryonic kidney cell line (HEK293T), as a negative control, were stained with the mice antisera and analyzed by flow cytometry (Figure 5C). This analysis revealed that the antibodies interact strongly with MCF-7 and T47D cells but not with the negative control cells. A similar outcome was obtained from confocal microscopy studies. The primary antibodies (those elicited by the vaccine) interact with the MUC1 present on the surface of cells MCF7 (Figure 5D, upper panel) and this interaction can be detected by a secondary anti-human antibody. This observation is not possible with HEK293T, which lack these glycoproteins on the surface. Significantly, the antigens elicited by the vaccine also positively stained cancer cells from biopsies of breast-cancer patients (Figure 5D, lower panel), but no staining is observed in case of cells from healthy patients. Thus, these results demonstrated the antigen mimic potential of unnatural glycopeptide comprising these two unnatural residues (***S*Thr*** and **fPro**).

2.3. The use of a Tn antigen that exhibits a glycan surrogate

In order to expand the number of Tn mimics, we prepared a derivative that presents a chemical modification in the sugar moiety. The new Tn analog carries a threonine linked to a mimic of GalNAc derived from a sp^2-iminosugar (Figures 1 and 3B-right panel).[61] This unnatural carbohydrate, which has demonstrated to afford chemically and enzymatically stable conjugates,[62,63] was incorporated onto the most immunogenic fragment of MUC1, and the resulting glycopeptide was studied in solution by following our well-stablished methodology, and in the solid state bound to the SM3 antibody. The conformational analysis in water revealed that the unnatural Tn scaffold adopts a geometry like that found for the natural Tn-glycopeptide, with the amino acid in an extended conformation and the glycosidic linkage showing the 'eclipsed' conformer as the most populated one (Figure 3B, right panel). Of note, the X-ray structure of the complex of this antigen with SM3 antibody revealed that the Tn surrogate favors the CH/π interaction between the methyl group of the sp^2-iminosugar and a tryptophan residue of the antibody. This experimental evidence could explain the higher affinity observed for the designed glycopeptide relative to the natural counterpart. The glycopeptide was then conjugated to the protein carrier Keyhole Limpet Hemocyanin (KLH)[64] and the subsequent formulation was tested in mice, following an immunization strategy similar to that used in the α-methylserine-containing vaccine, but using complete and incomplete Freund's adjuvant in the different steps of the process. To our delight, the unnatural vaccine was able to elicit in mice higher levels of IgG antibodies relative to the natural one containing the **Tn-Thr** antigen. As in previous cases, the antibodies elicited by the unnatural vaccine can selectively recognize native tumor associated MUC1 presented on the surface of human cancer cells, as can be deduced form the flow cytometry and confocal microscopy studies (Figure 6C).

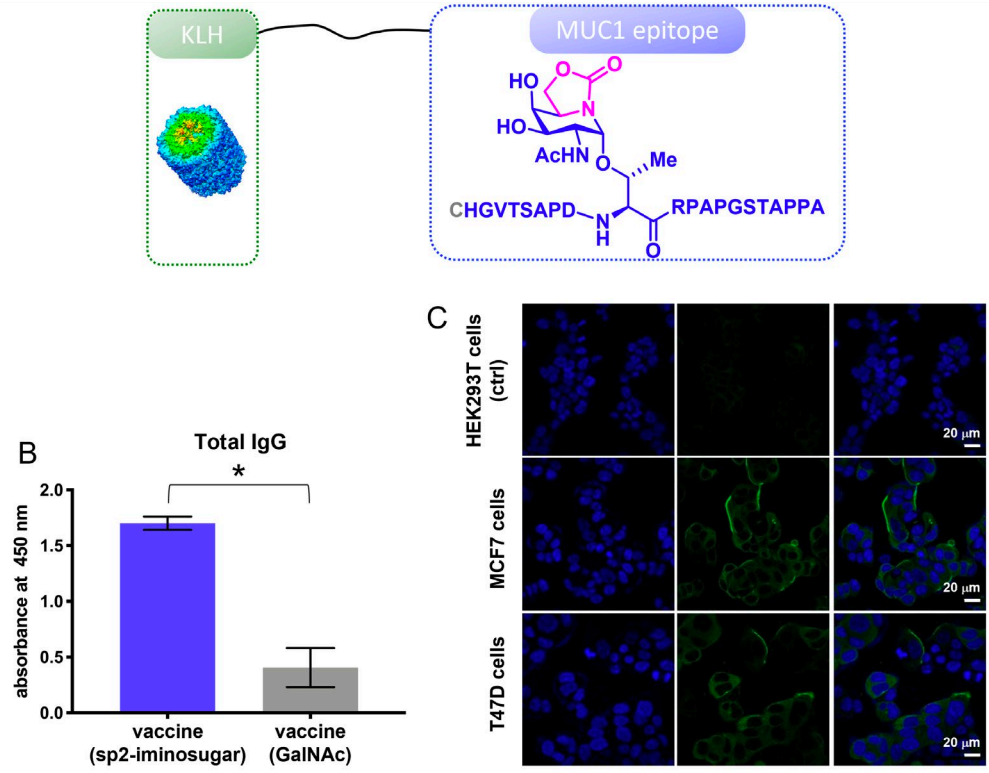


Figure 6. (A) Schematic representation of the anti-cancer vaccine based on a MUC1-glycopeptide featuring the sp^2-iminosugar conjugated to KLH protein. (B) Total IgG anti-MUC1 antibody titrations after immunization with either vaccine bearing the sp^2-iminosugar or the natural **Tn-Thr**. ELISA plates were coated with a natural MUC1-like glycopeptide. The horizontal lines indicate the mean for the group of mice (n = 3). An asterisk indicates a statistically significant difference (*P < 0.05). (C) Confocal microscopy images show that mice antisera after vaccination with the unnatural vaccine stain breast cancer cells MCF7 and T47D expressing tumor associated MUC1, but not those that do not express tumor associated MUC1 on their surface, HEK293T. Blue = Hoechst (nuclei); green = secondary antimouse IgG Alexa 488.

CONCLUSIONS

MUC1 is an overexpressed glycoprotein in cancer cells and displays aberrant glycosylation patterns recognized by the immune system. Therefore, it has become a priority molecule for the development of cancer vaccines. Synthetic chemistry allows to include artificial moieties to investigate critical elements and enhance the immune response. This study presents the results obtained in mice of three different vaccines engineered by our group. The chemical changes cover adjustments of the peptide moiety, the glycosidic linkage, and the sugar moiety. The *in vivo* results are confronted with the conformational space sampled by the new surrogates. Those derivatives that mimicry the conformational behavior of the Tn antigen (GalNAcα-*O*-Thr), that is, stiff glycosidic linkage and peptide side chain, could foster a more robust immune response. The results reviewed here can provide key information for novel routes and opportunities for modular construction of antitumoral vaccines.

CONFLICT OF INTEREST

All authors declare non-conflict of interest.

ACKNOWLEDGEMENTS

We thank the *Agencia Estatal de Investigación* of Spain (AEI; project RTI2018-099592-B-C21), and the EU (Marie-Sklodowska Curie ITN, DIRNANO, Grant Agreement No. 956544). A. Asín thanks the *Asociación Española Contra el Cáncer en La Rioja* for a predoctoral fellowship. F. G.-M. acknowledges the contract "Beatriz Galindo" from the Ministry of Universities of Spain.

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