Immunotherapy for Cancer: Synthetic Carbohydrate-based Vaccines

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Abstract

Aberrant glycosylation of glycoproteins and glycolipids of cancer cells, which correlates with poor survival rates, is being exploited for the development of immunotherapies for cancer. In particular, advances in the knowledge of cooperation between the innate and adaptive system combined with the implementation of efficient synthetic methods for assembly of oligosaccharides and glycopeptides is providing avenues for the rationale design of vaccine candidates. In this respect, fully synthetic vaccine candidates show great promise because they incorporate only those elements requires for relevant immune responses, and hence do not suffer from immune suppression observed with classical carbohydrate-protein conjugate vaccines. Such vaccines are chemically well-defined and it is to be expected that they can be produced in a reproducible fashion. In this review article, recent advances in the development of fully synthetic sub-unit carbohydrate-based cancer vaccines will be discussed.

1. Introduction

Traditional treatment options for cancer such as surgery, chemotherapy and radiation, are often unselective causing many unwanted side reactions and may not be able to neutralize cancer cells that have metastasized. To overcome these problems, efforts are directed towards harnessing the power of the innate and adaptive immune system to selectively remove malignant cells. Immunotherapy, albeit in an unspecific form, was first employed over 100 years ago, long before the intrinsic mechanisms of cancer immunology were understood.[1] It was found that a mixture of bacterial toxin injected into a tumor mounted an immune response in patients that led to its complete eradication. Several immunological mechanisms have been proposed for Coley's observation and one rationale pinpoints the endotoxins in the bacterial mixture as the active substances, inducing the release of cytokines such as TNF-α and interleukins, leading to the activation of macrophages, natural killer cells and cytotoxic T cells. Our current understanding of the molecular mechanisms of the innate and adaptive immune system has stimulated intense research in the rationale development of immunotherapies for cancer.[2, 3]

Classical vaccines are employed prophylactically to provide protection against infectious diseases. Most experimental cancer vaccines, on the other hand, are used therapeutically to evoke an immune response capable of eradicating an already existing disease.[4–7] Also, a cancer vaccine can be used to treat minimal residual disease and to protect against relapses once a tumor has been de-bulked by surgery or chemotherapy. Experimental cancer vaccines pursued today can be categorized as whole cell vaccines, antigen specific vaccines, dendritic cell vaccines and viral vectors and DNA vaccines.[2]

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The first efforts to develop a cancer vaccine were based on the use of the patient's tumor cells, which after removal were inactivated by for example irradiation, and then re-injected into the patient. By using a patient's own tumor cells, the immune response is expected to be tumor-specific and therefore should not affect normal cells.[8] Another advantage of whole cell vaccines is that there is no need to identify tumor specific antigens. Since its introduction, this technology has been refined and tumor cells, autologous and allogeneic, are now genetically modified to express high levels of appropriate co-stimulatory proteins to ensure that they are primed for tumor-cell removal.[9] However, major drawbacks of this approach include the labor intensiveness and cost of such personalized medicine (in the case of autologous cells), and the difficulties of measuring specific immune responses.

The identification of tumor-associated carbohydrate antigens (TACA) has made it possible to develop antigen-specific vaccines. Such vaccines offer the distinct benefit of providing methods for monitoring and evaluating specific immune responses. For example, for over four decades, it has been known that the majority of human cancers are characterized by aberrant glycosylation.[10–13] Tumor cells may over-express truncated versions of oligosaccharides, unusual terminal oligosaccharide sequences, and an increased sialylation of cell-surface glycolipids and O- and N-linked glycoproteins. Several mechanisms have been proposed for the formation of TACAs, such as altered metabolism of tumor cells, changes in the tumor environment, and consequent changes in the expression of multiple genes of the glycosylation machinery.[14–16] A truncated oligosaccharide of a glycoprotein may render a part of the peptide backbone that is normally shielded by the glycan more accessible to the immune system. Apart from being membrane bound, many tumor-associated carbohydrate antigens (TACAs), are secreted into the serum by the tumor-cells. Thus, these antigens provide viable targets for the development of both diagnostic and tumor-selective or tumor-specific carbohydrate-based vaccines.[17–26]

2. Tumor-associated carbohydrate antigens (TACAs)

Protein- and lipid-bound oligosaccharides found on the surface of cells are involved in many essential processes impacting eukaryotic biology and disease, and thus it is not surprising that malignant cells, which display differences in cell adhesion and cell motility, also display altered cell surface glycosylation.[27–29] The abnormal glycosylation has been shown to play a key role in the induction of invasion and metastasis and there is a wealth of evidence that abnormal glycosylation in primary tumors is closely correlated with the survival rate of cancer patients.[30]

Tumor-associated carbohydrates can be linked to lipids such as gangliosides, or to proteins such as mucins. Glycolipid TACAs includes GM2, GD2, GD3, fucosyl-GM1, Globo-H, and Lewis^y (Le^y) and the glycoprotein TACAS include the truncated Tn-, TF and sialylated Tn (STn)- antigens as well as Globo-H and Le^y (Figure 1).

The glycosphingolipids GM2, GD2, and GD3 are implicated in human melanomas and have been the target of extensive vaccine research.[17] Although detectable on normal cells, they are highly expressed on malignant cells. Globo-H, also known as the MBr-1 antigen,[31] was isolated from human breast cancer cells using a monoclonal antibody MBr-1,[32, 33] and has since also been identified as a tumor- associated antigen for ovary, colon, prostate, lung and small-cell lung cancers.[34]

Several tumor-associated glycosphingolipids have been identified as adhesion molecules and, consequently, these compounds have been shown to promote tumor-cell invasion and metastasis.[35] For example, the Lewis antigens sialyl Lewis^a (SLe^a), SLe^x, SLe^x-Le^x, and Le^y are identified as human tumor-associated antigens (Figure 1).[36, 37] The Le^y tetrasaccharide is over-expressed on a range of carcinomas including ovary, breast, colon, prostate, and non-

small cell lung cancers. The KH-1 antigen, which displays the heterodimeric Le^y-Le^x heptasaccharide, was isolated from human colonic adenocarcinoma cells.[38] This antigen has only been found on the surface of these cells and has never been isolated from normal colonic tissue, thus providing a highly specific marker for malignancies.[39, 40]

The blood group precursors, Tn, STn, and TF-antigens, are the result of incomplete *O*-glycan synthesis. The Tn-antigen, αGalNAc-Thr/Ser, results from the lack of core 1 β3galactosyltransferase (T-synthase). Recently, it has been shown that the expression of T-synthase is regulated by a key molecular chaperone, Cosmc, which resides in the ER. Mutations that leads to loss of function of Cosmc, lead to loss of T-synthase activity.[41, 42] The antigens are not exposed in normal tissue but are found immuno-reactive in a majority of carcinomas, thus representing excellent targets for cancer vaccine development. Mucins, which are a family of densely glycosylated high molecular weight proteins, are implicated in epithelial cancers. [43] Mucins also serve as diagnostic tools for cancers. For example, MUC-1, which is a membrane-bound mucin, is found over-expressed in more than 90% of breast carcinomas[43] and is also found in patient sera and have found clinical use as a marker for breast cancer.[44] In addition, MUC-1 is associated with ovarian, lung, colon, and pancreatic carcinomas.[43] The over-expressed tumor-associated MUC-1 display the truncated antigens Tn, STn, and TF due to deficient glycosylation in addition to providing a scaffold for the Lewis antigens and the Globo series. The same TACAs can thus be attached to both mucin and non-mucin aglycons on the same malignant cell.

3. Difficulties associated with carbohydrate vaccine development

Although tumor-associated carbohydrates offer promise as cancer vaccines, many issues complicate their use. Firstly, the carbohydrate antigen of interest needs to be available in sufficient quantities, high purities and structural integrity. However, isolation of the antigen from natural material is a task of Herculean proportions due to the heterogeneity of cell-surface glycosylation. Synthetic organic chemistry presents, however, a viable solution to this problem and can provide homogeneous oligosaccharide antigens in high purity and undisputable structural integrity in relatively large amounts. The continuing improvements in methods for oligosaccharide synthesis have equipped organic chemists with more sophisticated tools, including one-pot syntheses[45–50] and automated oligosaccharide synthesis.[51, 52]

Secondly, augmenting an immune response against carbohydrates is associated with difficulties owing to their inherently T-cell independent nature. In this respect, responses to this class of antigen is markedly different from response to proteins and peptides, and often elicits only a short-lived low affinity IgM antibody response, which lack memory and do not induce a T-cell response. This feature has hampered the development of carbohydrate- and glycopeptide-based vaccines. In addition, as the TACAs are regarded as "self-antigens", since they may be present on normal cells, albeit in low concentration, they receive tolerance from the immune system and their antigenicity is low. The TACAs are often shedded into the blood-stream by the growing tumor which further reinforces immuno-tolerance. As a consequence, induction of high affinity IgG antibodies against TACAs has proven much more challenging than the induction of similar antibodies against viral and bacterial carbohydrates antigens. Indeed, high titers of IgG antibodies have been referred to as the "holy grail" in carbohydrate-based tumor vaccinology.[53] Major research efforts have been focused to break this immuno-tolerance by better presenting TACA antigens so as to induce specific and relevant antibody responses. After a brief description of oligosaccharide synthesis and the immune response to carbohydrates, we will detail some of the research efforts directed towards the development of carbohydrate-based cancer vaccines.

4. Immune response to carbohydrates

It has been shown that antibodies that target tumor-related carbohydrate and glycopeptide antigens have the ability to eliminate circulating tumor cells.[5, 54, 55] These antibodies can be acquired by passive immunization, (*i.e.* immunization with the antibody itself), or by active immunization with a vaccine that contains the carbohydrate epitope. The antibodies can also be acquired naturally and for example, for melanoma patients, detectable levels of natural antibodies against the ganglioside GM2 correlate with improved survival.[56]

Antibodies against tumor-associated carbohydrates can mediate elimination of tumor cells by complement-dependent cytotoxicity (CDC) and/or by antibody-dependant cellular cytotoxicity (ADCC) performed by NK cells and macrophages. The antibodies also have been shown to interfere with receptor-mediated signaling, adhesion, and metastasis.

Antibodies are produced by B-cells that have been activated with their cognate antigen. The B-lymphocytes carry membrane-bound Ig proteins that can recognize a wide variety of compounds. Carbohydrates, for example, can bind to receptors of B-lymphocytes, induce cross-linking of the Ig proteins, which will lead to activation of the B-cell and production of low affinity IgM antibodies.[57] To achieve a class switch to high affinity IgG antibodies, the B-cells need to interact with helper T-cells (Figure 2).[58, 59] Activation of helper T-cells requires, in turn, the involvement of antigen-presenting cells (APCs). The most highly specialized APCs are dendritic cells, which are capable of capturing protein antigens that, after internalization and proteolytic cleavage into peptides, are presented on the surface of the APC as a complex with class II MHC molecules. Subsequently, the APCs will migrate to lymphoid organs where the peptide complexed to class II MHC peptide will interact with the T-cell receptors of naïve T-lymphocytes, resulting in their activation.[60, 61] A similar type of interaction via MHC class II exists between B-cells and T-cells. Naïve B- and helper T-cells reside in different compartments of the lymphatic system and are induced to migrate towards one another only after activation by an antigen ensuring, that the cells come together only when needed. Thus, activation of naïve T-cells induce migration to the T-cell zone where the T-helper cell will interact with B-cells.[62] The class II MHC-peptide complex presented by a B-cell will mediate an interaction with the helper T-cells, which will lead to expression of co-stimulatory proteins, further augmenting the interaction between the two cell types. Activated helper T-cells express CD40L, which will bind with CD40 on the B-cell resulting in cytokine production by the T-cell.[63] A combination of binding to CD40 and cytokine signaling will stimulate the B-cell to proliferate and differentiate into antibody-secreting cells. In addition, memory B-cells will be formed that live for a long time and respond rapidly to subsequent exposures of antigen by differentiating into high-affinity (IgG) antibody secretors.[64]

In addition to activation of B and T lymphocytes, adaptive immune responses require danger signals that are provided by the innate immune system. In the vaccine setting, an adjuvant is included to provide the necessary signals for APC maturation and cytokine release. The discovery of Toll-like receptors (TLRs) less than a decade ago has advanced our understanding of early events in microbial recognition and response and the subsequent development of an adaptive immune response.[65, 66] There is emerging evidence that cytokines, produced by activation of TLRs through their interaction with adjuvants, play crucial roles in the initiation and control of the adaptive immune response.[67−69] The cytokines stimulate the expression of a number of co-stimulatory proteins such as CD28 for optimum interaction between T-helper cells and B- and antigen presenting cells. In addition, some cytokines and chemokines are responsible for overcoming suppression mediated by regulatory T-cells. Other cytokines are important for directing the effector T-cell response towards a T-helper-1 (Th-1) or T-helper-2 (Th-2) phenotype.

5. The classical approach for carbohydrate-based vaccine development using protein conjugates

Classical carbohydrate-based cancer vaccines follows the successful approach used for bacterial carbohydrate antigens,[70] involving the conjugation of a carbohydrate antigen to a carrier protein such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid (TT).[20] The carrier protein incorporates helper T-epitope peptides, which are presented on the surface of an APC in complex with MHC after internalization and proteolysis. The protein carrier, thus enhances the presentation of the carbohydrate antigen and induces activation of helper T-cells. Proteins can also possess mitogenic and adjuvant-like properties that stimulate the innate immune response to provide cytokines. In addition, conjugate vaccines are often administered with an immuno-adjuvant such as BCG, Detox, QS-21, GPI-0100 or MPLA, to further stimulate the innate immune response.

An important issue for carbohydrate conjugate vaccine development is the use of appropriate conjugation chemistry to attach the carbohydrate antigen to the carrier protein. Carbohydrates isolated from natural sources are typically conjugated to a protein carrier by reductive amination through the aldehyde functionality of the reducing end sugar. This may destroy vital recognition elements, especially in the case of short oligosaccharides, resulting in a decrease or complete loss of immunogenicity. Synthetic oligosaccharides, on the other hand, can be designed to incorporate a linker that has a functional group with unique reactivity for selective conjugation to a carrier protein in a manner that does not interfere with the antigenic epitope. The choice of protein carrier, adjuvant, and linker chemistry can greatly influence the immune response to the weakly immunogenic tumor-associated carbohydrate antigens.[71–74] As will be discussed below, the linker, for example, can abrogate the immune response to the carbohydrate antigen. Early work in the carbohydrate-protein conjugate cancer vaccine field involved isolated gangliosides GD2, GD3, and GM2.[17] Helling and coworkers established that improved immune response can be achieved in mice by linking the tumor-associated ganglioside GD3 to a carrier protein and administer the vaccine candidate with an adjuvant.[75, 76] It was found that the choice of carrier protein, method of conjugation, and the nature of the adjuvant greatly influenced the immune response. The best response was achieved when KLH was used as carrier protein together with co-administration of the adjuvant QS-21. This protocol elicited both IgM and IgG anti-GD3 antibodies that could induce complement-mediated lysis of human melanoma cells expressing GD3.

Based on these findings, several clinical trials have been conducted with ganglioside-KLH conjugates of which the GM2-KLH vaccine has shown most promise.[17] The GD3-KLH conjugate on the other hand, failed to raise an antibody response in humans.[77] In attempts to increase the immunogenicity of the GD3 epitope, a GD3 lactone was synthesized and conjugated to KLH, which led to a slight improvement of antigenicity.[78] The modified GD3 induced antibodies that were able to recognize natural GD3. These results highlight an important advantage of organic synthesis of tumor-associated carbohydrate antigens; structural modifications can be made to can improve immunogenicity.

5.1 Conjugate vaccines using synthetic carbohydrate antigens

The power of organic synthesis has also made it possible to prepare highly complex tumor-associated carbohydrate antigens. Efficient synthetic methods are critical for the development of carbohydrate-based cancer vaccines and although considerable improvements have been made in this field,[52, 79–86] the construction of oligosaccharides and glycopeptides remains a challenge task due to the combined demands of elaborate procedures for glycosyl donor and acceptor preparation and the requirements of regio- and stereo-selectivety in glycoside bond formation. Many new leaving groups for the anomeric center have been developed, which can

be introduced under mild reaction conditions and are sufficiently stable for purification and storage for a considerable period of time. The most commonly employed glycosyl donors include anomeric fluorides,[87] trichloroacetimidates,[88] and thioglycosides[89]. These approaches, under the appropriate reaction conditions, can give high yields and good anomeric ratios. The glycal assembly strategy,[90] the use of anomeric sulfoxides,[91] and dehydrative glycosylation protocols[92–97] are also emerging as attractive tools for the assembly of complex oligosaccharides. Furthermore, these leaving groups can be activated under mild reaction conditions and guarantee high yields and good anomeric ratios when performed under the appropriate reaction conditions. Convergent synthetic strategies that allow the convenient assembly of complex oligosaccharides from properly protected building units involving a minimum number of synthetic steps have become available. In particular, one-pot multi-step approaches for selective monosaccharide protection[45, 46] and oligosaccharide assembly are being pursued, which do not require intermediate work-up and purification steps and hence speed-up the process of chemical synthesis considerably. Several research groups have demonstrated that chemoselective, orthogonal and iterative glycosylation strategies, which exploit differential reactivities of anomeric leaving groups, allow several selected glycosyl donors to react in a specific order resulting in a single oligosaccharide product.[47–49, 98–100] Methods for solid phase oligosaccharide synthesis have been reported and these procedures shorten oligosaccharide synthesis by removing the need to purify intermediate derivatives.[51, 101]

A crucial step in the chemical synthesis of glycopeptide vaccine candidates is the merger of carbohydrate and peptide chemistry.[85, 86] Different synthetic approaches can be envisaged for the preparation of glycopeptides. For example, a protected (or unprotected) oligosaccharide can be linked to the side-chain of an amino acid and then be incorporated by solid phase glycopeptide synthesis. Alternatively, an unprotected oligosaccharide equipped with a proper functional group can be conjugated to a peptide using well-established conjugation chemistry, such as disulfide and thioether formation, and oxime chemistry (Table 1). Recently, native chemical ligation[102] and "click-chemistry"[103] (Cu(I)-mediated Huisgen cyclo-addition)[103, 104] have emerged as powerful tools for chemo-selective ligations. In the Huisgen cyclo-addition, an azide and an alkyne group reacts, typically in the presence of Cu(I), to form a triazole moiety (Table 1). Although attractive, it should be noted that the click-reaction introduces a rigid triazole moiety, which may be immunogenic and thus further suppress the low immunogenicity of a tumor-associated carbohydrate antigens. Native chemical ligation (NCL), on the other hand, is a chemo-selective reaction that results in the formation of an amide bond (Scheme 1).

Research teams led by Livingston and Danishefsky at Memorial Sloan-Kettering Cancer Center have made notable contributions to the field of carbohydrate-based cancer vaccine development, but several other research groups have reported elegant syntheses and immunological evaluations of these antigens.[50, 52, 73, 105–120] The Livingston-Danishefsky team have reported the synthesis Globo-H,[121] Lewis[y,122–124] Lewis[x,125] Lewis[b,122, 126] KH-1,[127] MUC-1,[128] and the Tn, STn and TF-antigens.[74, 129] Several of the antigens have also been synthesized in a clustered configuration in an attempt to improve immunogenicity. The rationale behind the clustered presentation of TACAs is that in the humoral immune response, after a B-cell recognizes its cognate antigen, antigen-induced clustering of the B-cell receptors is necessary to deliver the biochemical signals to the B-cell to initiate the process of activation.

In those cases, the oligosaccharide antigens were equipped with an allyl linker that after ozonolysis provided an aldehyde group, which allowed conjugation to the protein carrier by reductive amination.[121–123] An alternative method involves the use of maleimide derivatized proteins that can be reacted with thiolated carbohydrate antigens.[71, 128, 130] The conjugates

have been evaluated in mice and typically both IgM and IgG antibodies were elicited, which were able to recognize natural epitopes expressed by tumor-cells and induce complement-mediated lysis of tumor-cells.

Our group has developed a solid support and solution phase synthesis of the Le^y,[73, 116] Le^x,[116, 131] and the KH-1(Le^y-Le^x)[119] antigens, in which the KH-1 antigen was equipped with an artificial aminopropyl spacer (Scheme 2). In addition to the orthogonal Fmoc, Lev, Troc,[116, 132] and silyl protecting groups, a *p*-(benzoyl)-benzyl goup was used as a novel anomeric protecting group. This protecting group could be selectively removed at a late stage in the synthesis, thus offering the benefit of enhanced flexibility. The approach provided easy access to a Le^y glycosyl donor (**8**) and a Le^x acceptor (**16**) that could be coupled in one key glycosylation to provide the heterodimeric Lewis antigen (**17**; Scheme 2). The KH-1 antigen derivatized with a thio acetyl was conjugated to KLH that had been activated with electrophilic 3-(bromoacetamido)propionyl groups. Immunizations of the conjugate in combination with the adjuvant QS-21 evoked a strong immune response against the heptasaccharide. Studies of the cross-reactivity revealed that the antibodies also recognized the terminal Le^y antigen, albeit with much lower titers. However, the antibody recognition of the reducing end Le^x trisaccharide moiety was low, clearly demonstrated that the raised antibodies recognized an epitope spanning the two Lewis antigen monomers. Our findings support the notion that that it may be possible to develop a tumor specific anti-cancer vaccine targeting carbohydrate antigens.

A number of carbohydrate protein conjugates have been examined in Phase I, II, and III clinical trials.[78, 133−144] The results reported to date indicate that the carbohydrate-conjugate vaccines are well-tolerated, do not induce auto-immune reactions, and appear most promising when used in a combination with a potent adjuvant such as the saponin QS-21, the immunomodulator cyclophosphamide,[145] and stem cell rescue.[146] A clear correlation between vaccine-induced antibody responses and clinical course after immunizations has been found. However, even when optimized immunization protocols were used, it was difficult to induce high titers of the high affinity IgG antibodies in most patients. The results of the pre-clinical and clinical studies indicate that many factors can influence the antigenicity of tumor-associated antigens conjugated to carrier proteins. The choice of carrier protein, conjugation method, the nature of the linker, carbohydrate-loading onto the protein, and immuno-adjuvant can greatly influence the magnitude and specificity of the elicited immune response.[71−75, 147]

5.2 Problems associated with carbohydrate-protein conjugate cancer vaccines

The attachment a carbohydrate to a carrier protein represents a problematic aspect of conjugate vaccine development. In general, the conjugation chemistry is difficult to control, and may results in conjugates with ambiguities in composition and structure and batch-wise variations of prepared glycoconjugates. As a general rule, a higher loading of a tumor-associated oligosaccharide antigen onto the protein induces a stronger immune response and thus batch variations in loading may be detrimental to the vaccine efficacy. In addition, the linkers that are employed for the conjugation of the carbohydrate to a carrier protein can be immunogenic leading to epitope suppression.[73, 148] For example, we found that the rigid cyclohexyl maleimide linker (Figure 3), which is often employed in conjugation chemistry because of its rapid and selective reaction with thiol-derivatives at near neutral pH, dramatically reduced the immune response of mice towards the Le^y antigen. It was found that mainly IgM and IgG anti-linker antibodies had been elicited.[73] In this study, the carrier protein KLH was activated with a maleimide linker and then reacted with the Le^y antigen derivatized with a thiol-linker. Higher titers of anti-Le^y antibodies were obtained when the smaller and more flexible 3-(bromoacetamido)propionate linker was used for protein activation and attachment of the

Le^y antigen. In this case, the immune response towards the linker was reduced which probably led to a considerably improved immune response of the Le^y antigen.

Another major drawback of using carrier proteins is that they are highly immunogenic in themselves and will inevitably elicit strong B-cell responses. This feature can lead to carrier-induced epitope suppression, which in particular is a problem when "self-antigens' such as tumor-associated carbohydrates are employed. As result, novel strategies have pursued to more efficient present a tumor-associated carbohydrate epitope to the immune system resulting in a class switch to IgG antibodies. In particular, attention has been focused on subunit vaccines, which are devoid of any unnecessary immunogenic components, comprising only of those element necessary for evoking an innate and humoral immune response, which results in a more focused and antigen specific immune response.

6. Fully synthetic carbohydrate-based cancer vaccines

6.1 Fully synthetic two-component vaccines

One approach to improve the presentation of a TACA to relevant immune cells is to attach the antigen to a receptor ligand that can target or activate appropriate immune cells. Mannosylation of antigens, for example, may result in selective targeting to antigen presenting cells that carries mannose receptors.[149]

Toll-like receptors (TLR) ligands, such as the lipopeptide Pam$_3$Cys, which is a TLR2 ligand, has been attached to TACAs. TLR activation by Pam$_3$Cys, leads to cytokine production, which in turn, activates dendritic cells, macrophages, and B-cells.[150–153] An example utilizing Pam$_3$Cys in this fashion was reported by Toyokuni *et al.* who covalently linked a dimeric Tn antigen to Pam$_3$Cys (Figure 4).[154, 155] Although low titers of IgG antibodies were elicited, the study showed that a small synthetic carbohydrate antigen could generate an immune response against the carbohydrate without a macromolecular carrier.

Danishefsky and co-workers have utilized a similar strategy, and several TACAs including monomeric Le^y, a trimeric cluster of Le^y,[72, 124, 156] and a trimeric Tn-antigen cluster (Figure 5) were attached to Pam$_3$Cys.[157] Mice immunized with the vaccine constructs elicited antibodies that recognized the natural epitope expressed by relevant cancer cell-lines. However, mainly IgM antibodies were detected and it was found that co-administration with the external immuno-adjuvant QS-21 did not induce a class switch to IgG antibodies. For the Tn-antigen trimeric cluster, it was found that the trimeric presentation of this antigen gave higher titers of antibodies, which displayed enhanced recognition of Tn-expressing cancer cells. These results highlight that a lack of a helper T-epitope, which is required to induce a class switch to IgG antibodies and affinity maturation, results mainly in the production of IgM antibodies.

A commendable chemical synthesis was undertaken to obtain a unimolecular multi-antigenic construct comprising the Globo-H, Le^y, STn, TF, and Tn antigens all attached to the same peptide backbone (Figure 6).[158, 159] The rationale of a polyantigenic construct[21, 160, 161] is that it combines TACAs that are closely related to a particular type of cancer, in this case prostate cancer. The oligosaccharides were synthesized using the glycal assembly method and equipped with pentenyl or allyl spacers, which subsequently were used to produce nor-leucine amino acid building blocks carrying the glycan on the side-chain. These building blocks were then used to synthesize the Pam$_3$Cys containing construct using conventional peptide chemistry. Mice were inoculated with the candidate vaccine in the presence of the adjuvant QS-21 and IgM antibodies against all antigens, were detected. When the multi-antigenic construct was linked to the carrier protein KLH and co-administered with QS-21 in a murine host, both IgM and IgG antibodies were elicited and the antibodies recognized three different tumor cell-lines all expressing two or more of the five antigens on their respective cell surfaces.

Two-component vaccines composed of a TACA and a CD4+ T-cell epitope have been designed and synthesized to enhance the interaction between the helper T-cell and B-cell thereby inducing higher titers of antibodies and achieving a class switch to IgG antibodies. In one attempt, a MUC-1 derived glycopeptide carrying a single STn moiety was linked to a CD4+ T-cell epitope derived from ovalbumin using a polar non-immunogenic linker (Figure 7).[162] The vaccine candidate was administered together with complete Freund's adjuvant, to transgenic mice expressing T-cell receptors specific for the ovalbumin T-epitope. It was found that an IgG antibody response was mounted and the concentration of serum antibodies increased after each boosts. It was also found that the antibodies were highly specific for the glycosylated MUC-1 peptide when compared to the unglycosylated MUC-1 peptide.

To target the heterogeneity in glycosylation of MUC-1 derived peptides, a construct containing three different B-cell epitopes, namely unglycosylated, Tn, and TF modified MUC-1 and one copy of the universal PADRE peptide helper T-epitope, was evaluated in mice (Figure 8).[163] IgG antibodies were raised towards all three B-cell epitopes and the antisera recognized native tumor epitopes expressed by human mammary adenocarcinoma cells.

A multi-antigenic glycopeptide (MAG) based on a non-immunogenic polylysine scaffold has successfully been pursued for eliciting antiboies agains the Tn antigen (Figure 9). A four arm lysine core with each arm extended by a CD4+ peptide T-helper epitope derived from Polio virus or the PADRE peptide and a trimeric Tn-antigen has been examined in mice and non-human primates.[164–167] The induced immune response promoted an increase in survival in tumor studies in mice, using both a prophylactic and therapeutic setting. In the therapeutic setting, administration of CY, which is reported to increase anti-tumor response, increased the survival rate from 40% to 80%.[166] The clustered MAG construct induced superior titers of anti-Tn IgG antibodies when compared to a KLH conjugate carrying trimeric Tn-clusters.[167] The MAG construct elicited good titers of IgG antibodies is the presence of the mild adjuvant alumn, whereas the clustered KLH conjugate required co-administration with the more potent adjuvant QS-21. Presentation of the TACA in a clustered mode, as in the MAG-conjugate, is ideal since after a B-cell recognizes its cognate antigen, antigen-induced clustering of the B-cell receptors is necessary to deliver the biochemical signals to the B-cell to initiate the process of activation.

6.2 Fully synthetic multi-component vaccines

A tri-component vaccine that contains a carbohydrate B-cell epitope, a helper T-cell epitope and a potent immune activator/modulator such as a TLR ligand or a cytokine would incorporate the minimal sub-units necessary to evoke an immune response against a carbohydrate.[168–170] In a first report, a fully synthetic three-component anti-cancer vaccine composed of the Tn-antigen, a helper T-epitope derived from *Neisseria meningitis*, and the TLR ligand Pam_3Cys was designed and synthesized, using a block synthetic approach.[169] The vaccine candidate was included in phospho-lipid based liposomes and then evaluated for its immunogenicity in mice, in the presence or absence of the external adjuvant QS-21. Although only low to moderate titers of IgG antibodies were raised against the Tn-antigen, the results indicated promising possibilities for further development of strategy.

In a subsequent study, two additional tri-component vaccine candidates composed of the tumor-related MUC-1 glycopeptide, a well-documented helper T-cell epitope from Polio virus, and either Pam_2CysSK_4 (**19**) or Pam_3CysSK_4 (**20**) as built-in immuno-adjuvants, were designed (Figure 10)[171, 172]. Pam_2CysSK_4 is a potent activator of TLR2 and TLR6, whereas Pam_3CysSK_4 induces cellular activation through TLR1 and TLR2[153]. Compound **19** was prepared by solid-phase peptide synthesis using a Rink Amide AM resin and conventional Fmoc-protected amino acid building blocks. After assembly of the glycopeptide, the acetyl esters of the saccharide moiety were cleaved by treatment with 80% hydrazine in methanol.

The lipid anchor, *N*-Fmoc-Pam$_2$Cys-OH, was coupled manually and after cleavage of the *N*-Fmoc group, the glycolipopeptide was cleaved of the resin and purified. Unfortunately, a similar linear synthesis of vaccine candidate **20** gave a product that was difficult to purify to homogeneity. Therefore, cancer vaccine **20** was prepared by liposome-mediated native chemical ligation of building blocks **24**, **25**, and **28** (Scheme 3). We recently found that the rate and yield of the NCL reaction was vastly improved if the reactants were embedded in liposomes.[172] This is especially the case for ligations using a hydrophobic reactant such as **28**, which has limited solubility in commonly used ligation buffers and solvents. In a typical protocol, a film of dodecylphosphocholine, thioester **24** and thiol **25** was hydrated in phosphate buffer (pH 7.5) in the presence of tris(2-carboxyethyl)phosphine and EDTA. The liposomes were sized by extrusion and the ligation was initiated by 2-mercaptoethane sulfonate (MESNa). The acetamidomethyl (Acm) thiol-protecting group was removed using Hg(II) acetate and then a second liposome-mediated NCL of deprotected glycopeptide **27** and lipopeptide thioester **28** gave Pam$_3$Cys-containing vaccine **20**. The vaccine candidates were incorporated into liposomes and their antigenicity studied in murine hosts. Compound **20** induced exceptionally high IgG antibody titers (Table 2). Further subtyping of the antibodies revealed high titers of IgG3 antibodies, which are typical for an anti-carbohydrate response, and a bias towards a Th2 response, as the levels of IgG1 antibodies were high. Co-administration with the external saponin immuno-adjuvant QS-21 did not alter the titers of IgG antibodies, however a shift towards a mixed Th1/Th2 response was induced. Interestingly, it was found that vaccine candidate **17**, which incorporates the TLR2 and TL6 ligand Pam$_2$CysSK$_4$ raised lower titers of anti-MUC-1 IgG antibodies. The elicited antibodies were shown to bind to MCF7 tumor cells, which express the MUC-1 antigen.

The influence of covalent attachment of the various components of the vaccine candidate on antigenic responses and the importance of the liposomal presentation of the vaccine were further investigated in mice (Figure 10). Uptake and proteolytic processing of antigen for subsequent presentation of a peptide-MHC class II complex on the cell surface of APCs is critical for eliciting IgG antibodies. It could be argued that by incorporating the three components into a liposome, proteolytic processing would be rendered unnecessary and thus a more robust immune response would be seen. However, it was shown that both the covalent attachment of the three components and the liposomal presentation were critical for achieving good antibody titers (Table 2). The lipid adjuvant moiety of the vaccine candidate facilitates the retention in the liposomes and aids in presenting the tumor-related antigen in a multivalent fashion to B-cell Ig receptors, which is required to be clustered to induce activation of B-cells. It was also shown that the TLR2 ligand Pam$_3$CysSK$_4$ induces cytokines, such as tumor necrosis factor-α (TNF-α), in a TLR2-dependent manner and facilitated uptake and internalization of the vaccine candidate by cells expressing TLR2. The covalent attachment of the lipid adjuvant thus also ensures that the cytokines are produced locally at the site where the vaccine interacts with relevant immune cells and facilitates uptake by APCs that express TLR2. The importance of TLR engagement was further investigated using a construct (**21**), containing an immunosilent lipopeptide anchor based on lipidated amino acids instead of the TLR agonist (Figure 10).[173] Lipidated glycopeptide **21** was synthesized in a straightforward manner using solid-phase peptide synthesis. The compound elicited significantly lower titers of IgG antibodies demonstrating that TLR engagement is critical for optimum antigenic responses. When compound **21** with the immunosilent lipid anchor was co-administered with Pam$_3$CysSK$_4$ (**22**) or monophosphoryl lipid A (**23**) similar titers of IgG antibodies were raised in mice. However, the resulting anti-sera had an impaired ability to recognize cancer cells.

Recently a multi-epitope vaccine consisting of a cluster of the Tn-antigen as a B-epitope, a CD4$^+$ T cell epitope, a CD8$^+$ T cell epitope, and a palmitic acid, serving as a built in adjuvant, was reported.[174, 175] The vaccine was based on the Regioselectively Addressable Functionalized Template (RAFT), which is a cyclic decapeptide consisting of proline, glycine,

and lysine residues. The side-chains of the lysine residues provide opportunities for selective incorporation of different antigens on opposite faces of the RAFT via classical ligation chemistry. The candidate vaccine was delivered in an adjuvant free setting and showed no adverse effects in a murine host. The elicited antibodies were shown to recognize human breast tumor cells MCF7 expressing the Tn-antigen. The vaccine also induced strong specific CD4^{+} T cell and CD8^{+} T cell responses. In prophylactic tumor studies with MO5 tumor cells, none of twenty mice developed a tumor in the monitoring period of 90 days. In contrast, the survival rate for mice immunized with a vaccine candidate lacking the palmitic acid adjuvant and CpG as an external adjuvant was determined to be 80%.

7. Conclusion

Fully synthetic anti-cancer vaccines targeting tumor-associated carbohydrates provide an attractive option for the treatment of cancer. Such vaccine candidates have major advantages as they can be designed to incorporate only those elements required for a desired immune response, and can be produced, in a reproducible fashion to give chemically well-defined compounds. Recent developments in the synthesis of complex carbohydrates and glycopeptides have made it possible to construct such glycoconjugate vaccine candidates for evaluation in pre-clinical and clinical settings. The research has provided important insight into which components influence, and are necessary, to evoke an immune response capable of eradicating tumor-cells. Recent reports have pointed out the importance of including TLR agonists in synthetic sub-unit vaccines that are capable of activating the innate immune system. Thus far, there are only two examples of fully synthetic multi-component vaccines that incorporate a tumor-associated glycopeptide antigen, a helper T-cell epitope and a built-in adjuvant that serves as a potent immune activator/modulator. It should also be mentioned that contrary to previous understanding it is now accepted that glycopeptides can mediate classical MHC-mediated immune responses. Thus, cytotoxic T lymphocytes (CTL), which, as opposed to helper T-cells, are expected to react with tumor cells, present an additional opportunity for glycopeptide-based cancer vaccines.[176] Native MUC-1 glycopeptides have been shown to bind to MHC class I molecules both *in vitro* and *in vivo*[177] and high affinity glycopeptides carrying the Tn- or TF-antigen have been used to induce a carbohydrate-specific cytotoxic T-cell response in mice.[178] Two-component vaccines, consisting of a CD8^{+} glycopeptide and a helper T cell epitope, have shown promising results in tumor models.[179] Although the results are promising, further pre-clinical and clinical research is necessary to assess the potential of these vaccine candidates to address their usefulness in cancer therapy.

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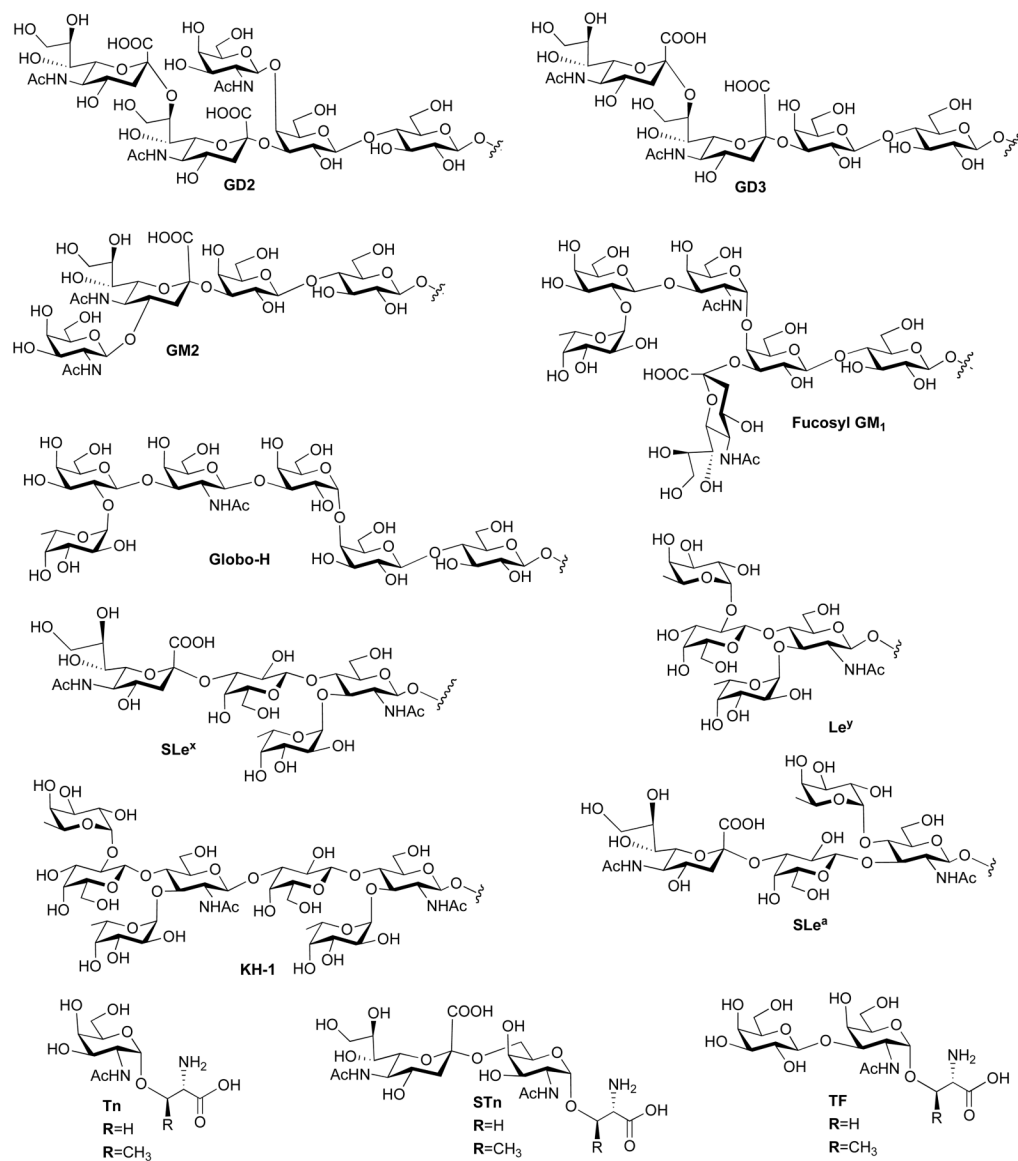
Figure 1.
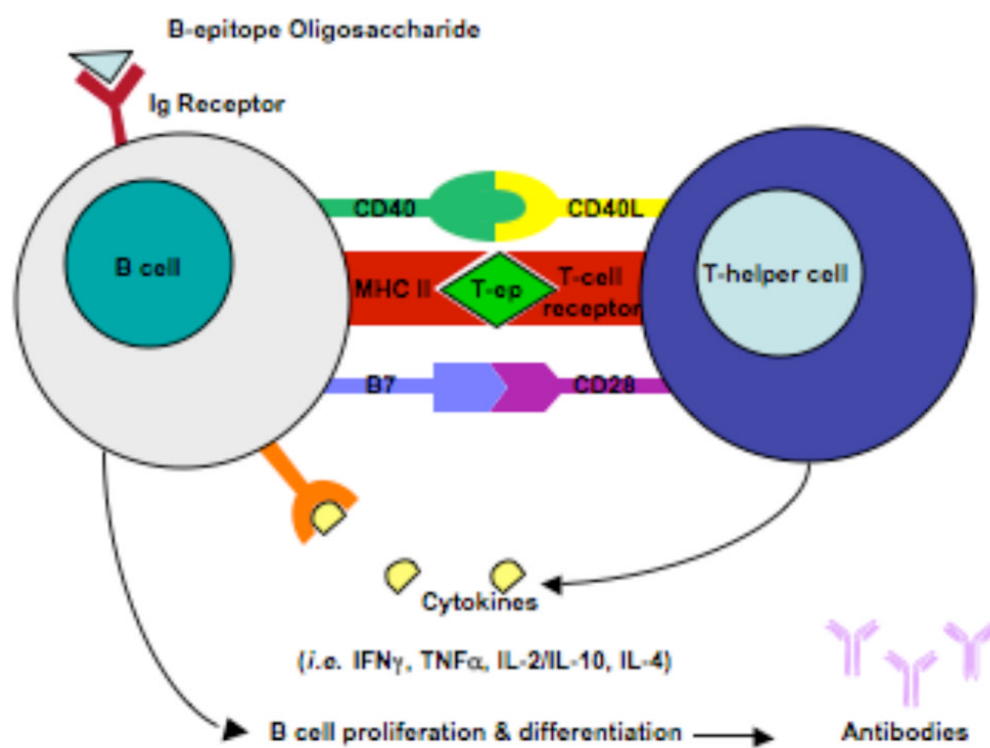Tumor-associated carbohydrate antigens.

Figure 2.
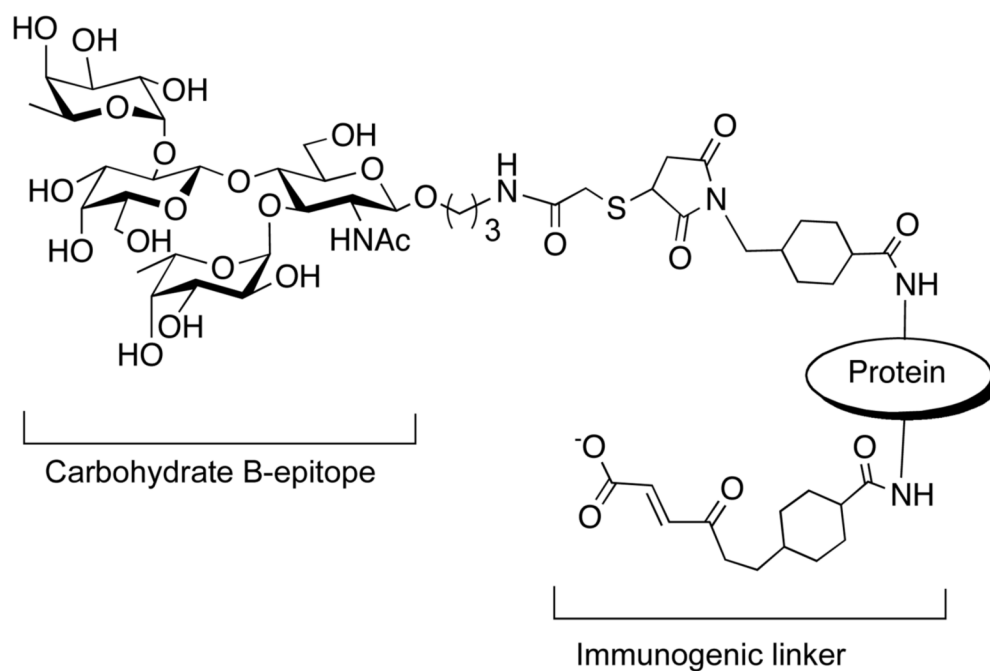Schematic presentation of the interaction between B cells and helper T cells

Figure 3.
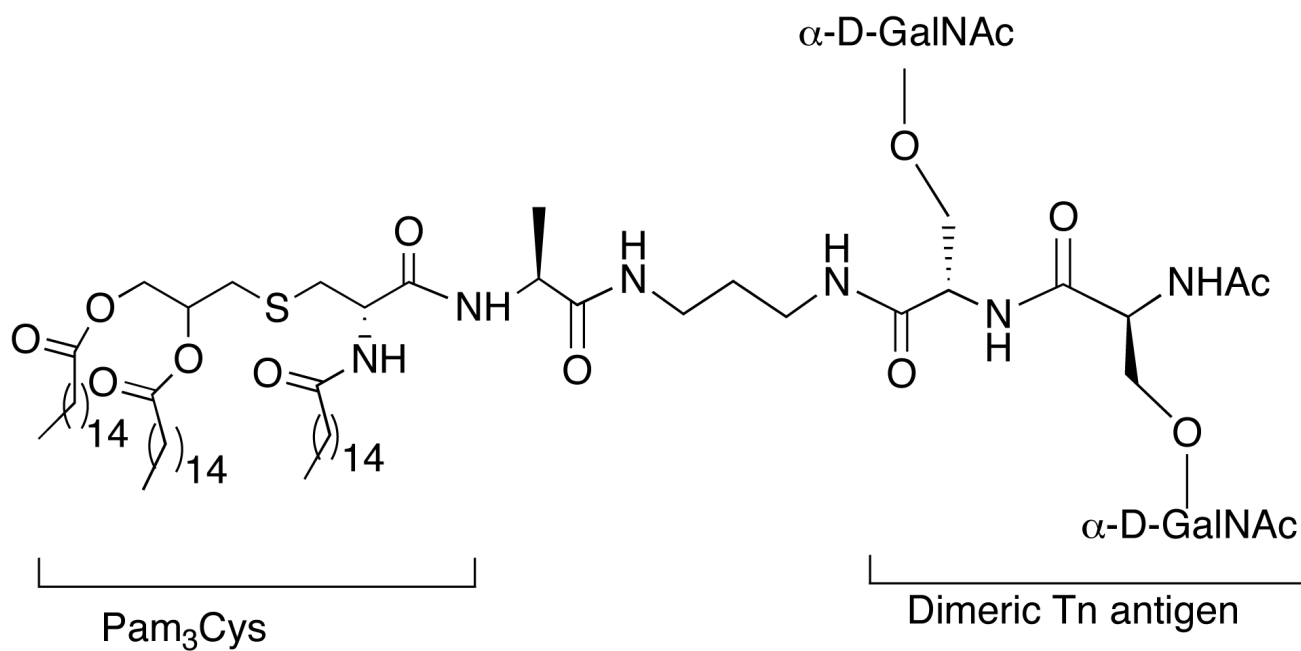The maleimide linker reacted with a thiol vs. unreacted hydrolyzed linker.

Figure 4.
A two-component cancer vaccine candidate consisting of a TLR ligand (adjuvant) and a dimeric Tn-antigen.
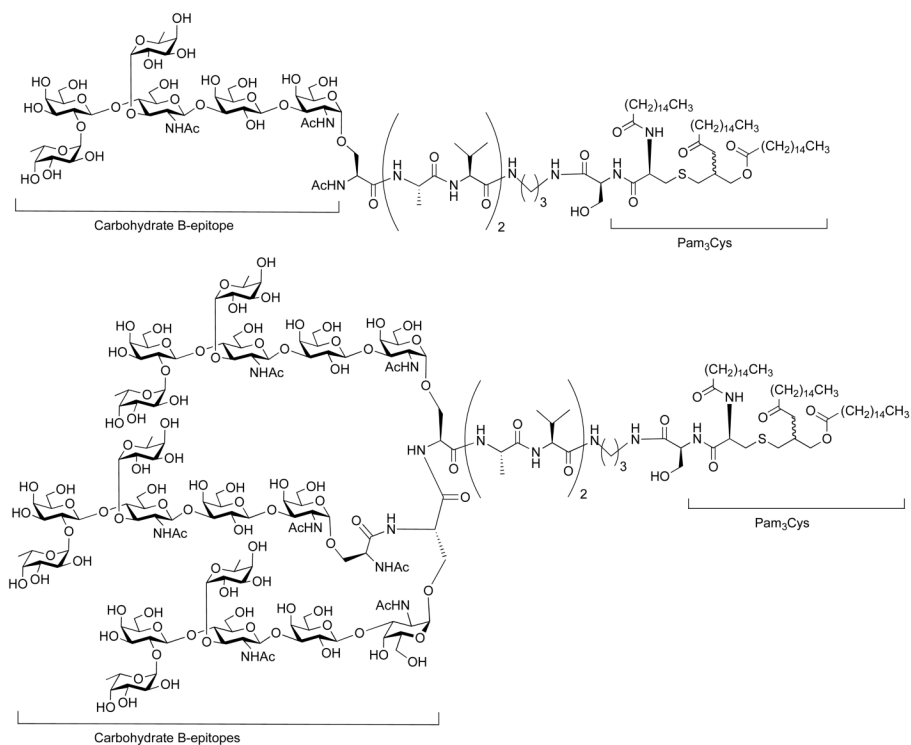
Figure 5.
Fully synthetic cancer vaccines incorporating the Lewis Y antigen and the TLR ligand, Pam$_3$Cys.
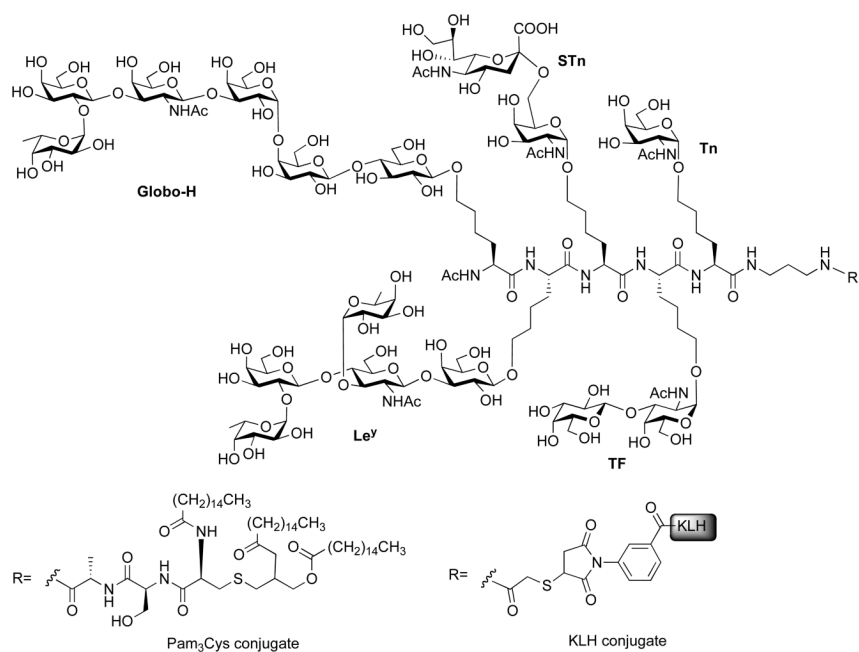
Figure 6.
Polyantigenic cancer vaccines.
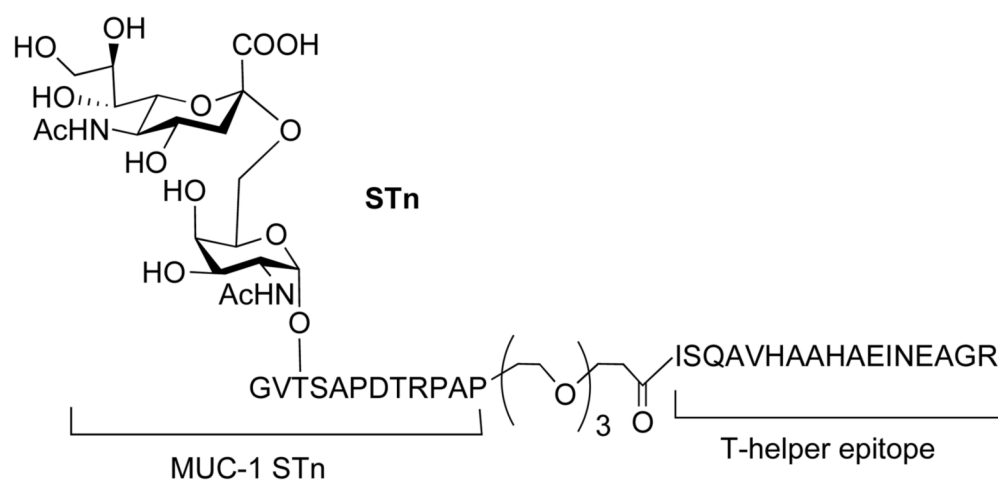
Figure 7.
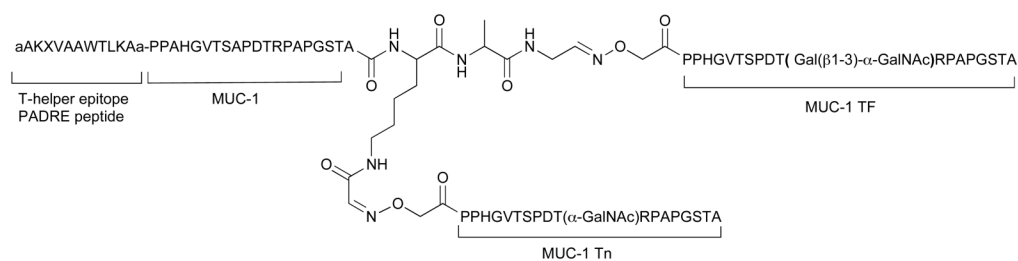A two-component cancer vaccine incorporating a glycopeptide B-cell epitope and a peptide helper T-cell epitope.
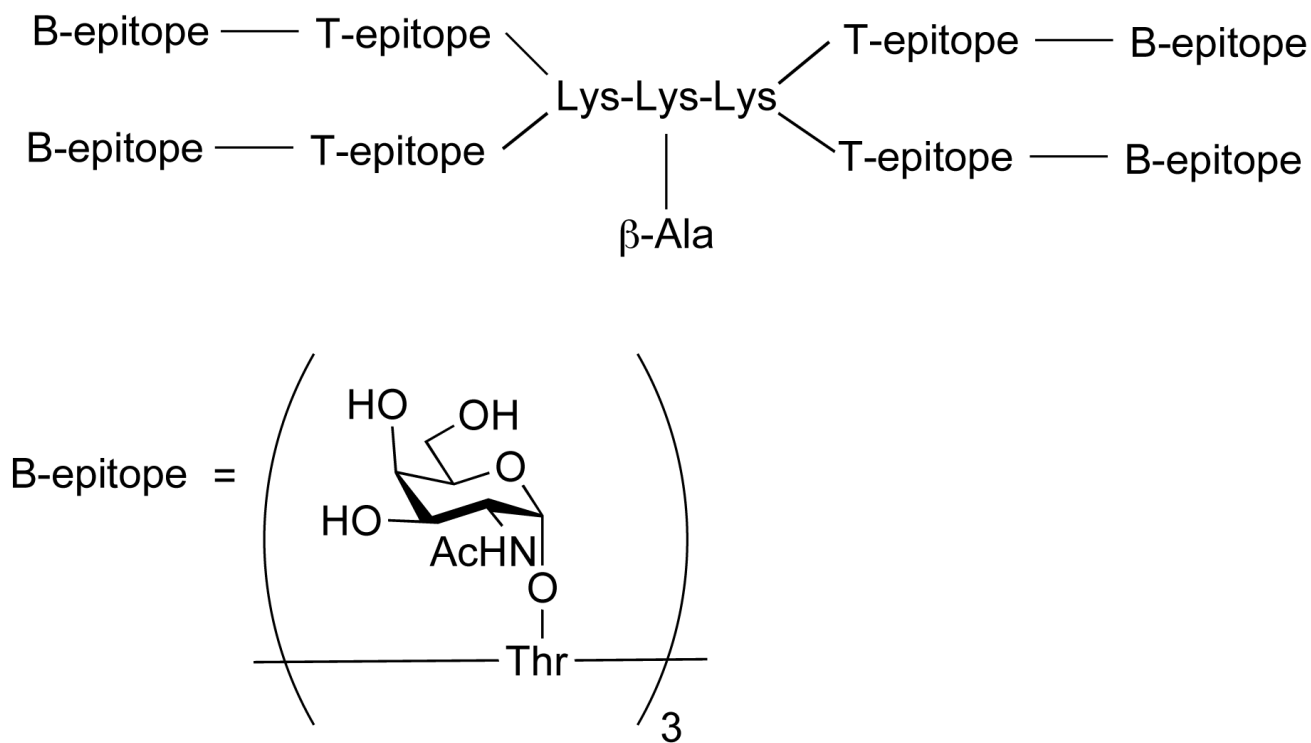
Figure 8.
A fully synthetic trimeric anti-cancer vaccine consisting of three B-cell epitopes and a helper T-cell peptide, PADRE.

B-epitope ⸺ T-epitope

B-epitope ⸺ T-epitope

Lys-Lys-Lys

T-epitope ⸺ B-epitope

T-epitope ⸺ B-epitope

β-Ala

B-epitope =

HO OH

HO

AcHN

O

O

Thr

3

T-epitope = KLFAVWKITYKDT or PADRE-peptide

Figure 9.
A multiantigenic glycopeptide based on an oligolysine scaffold.
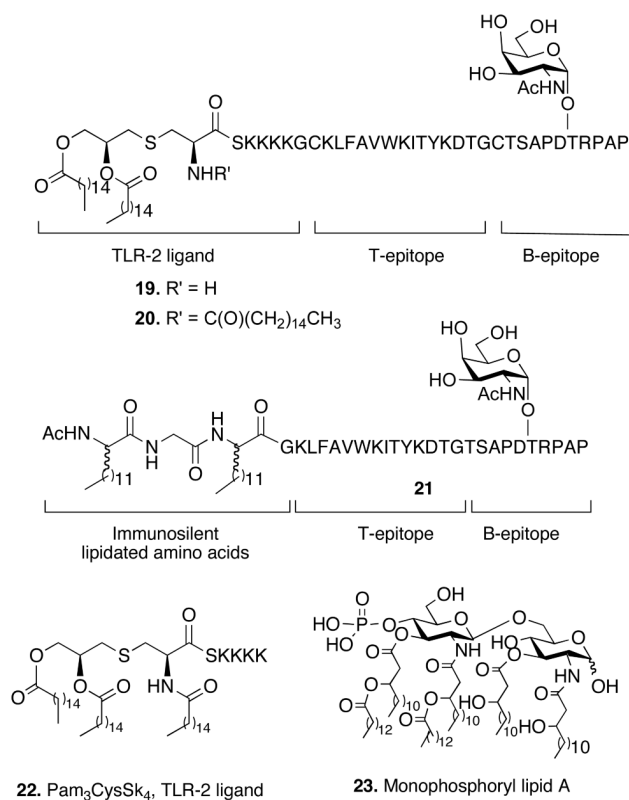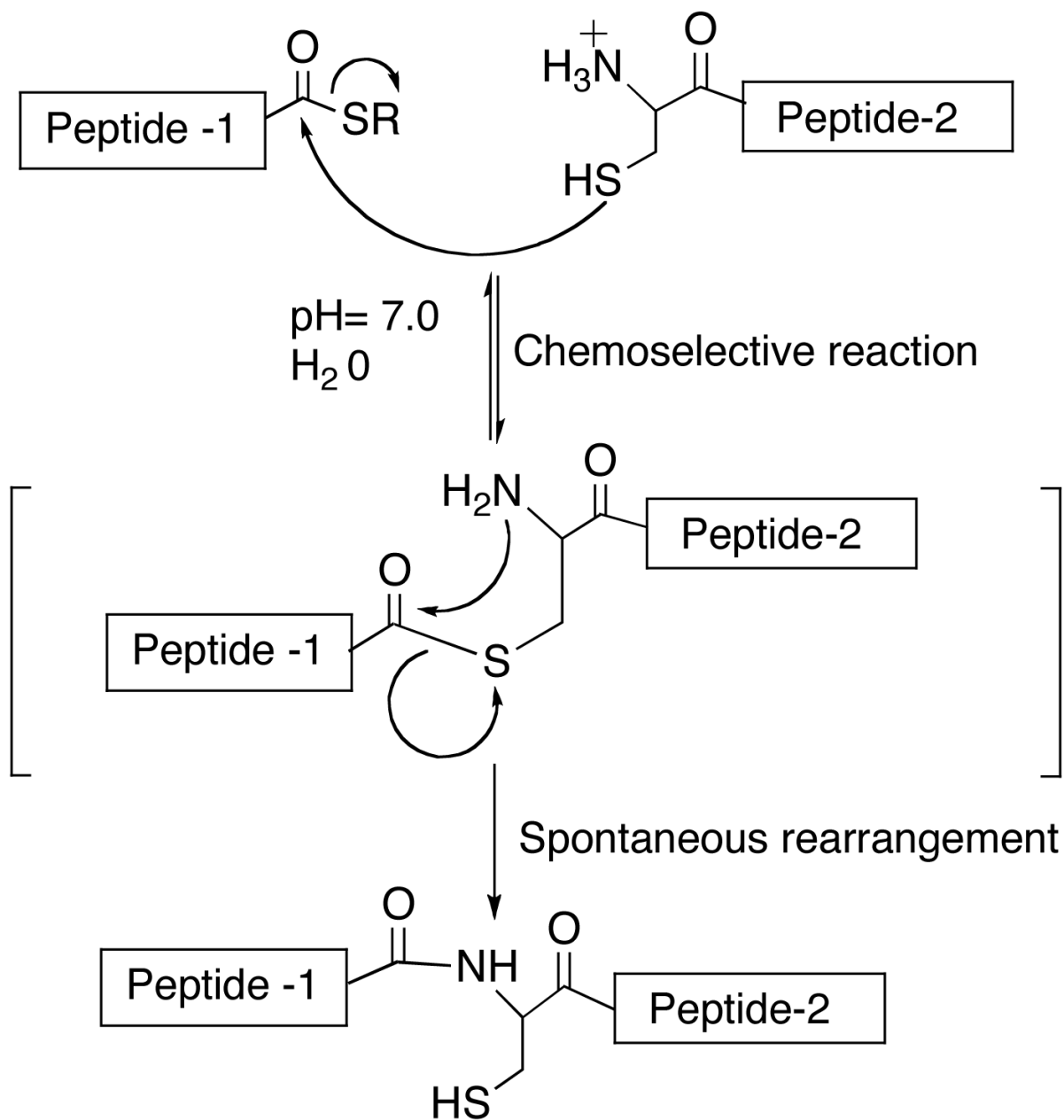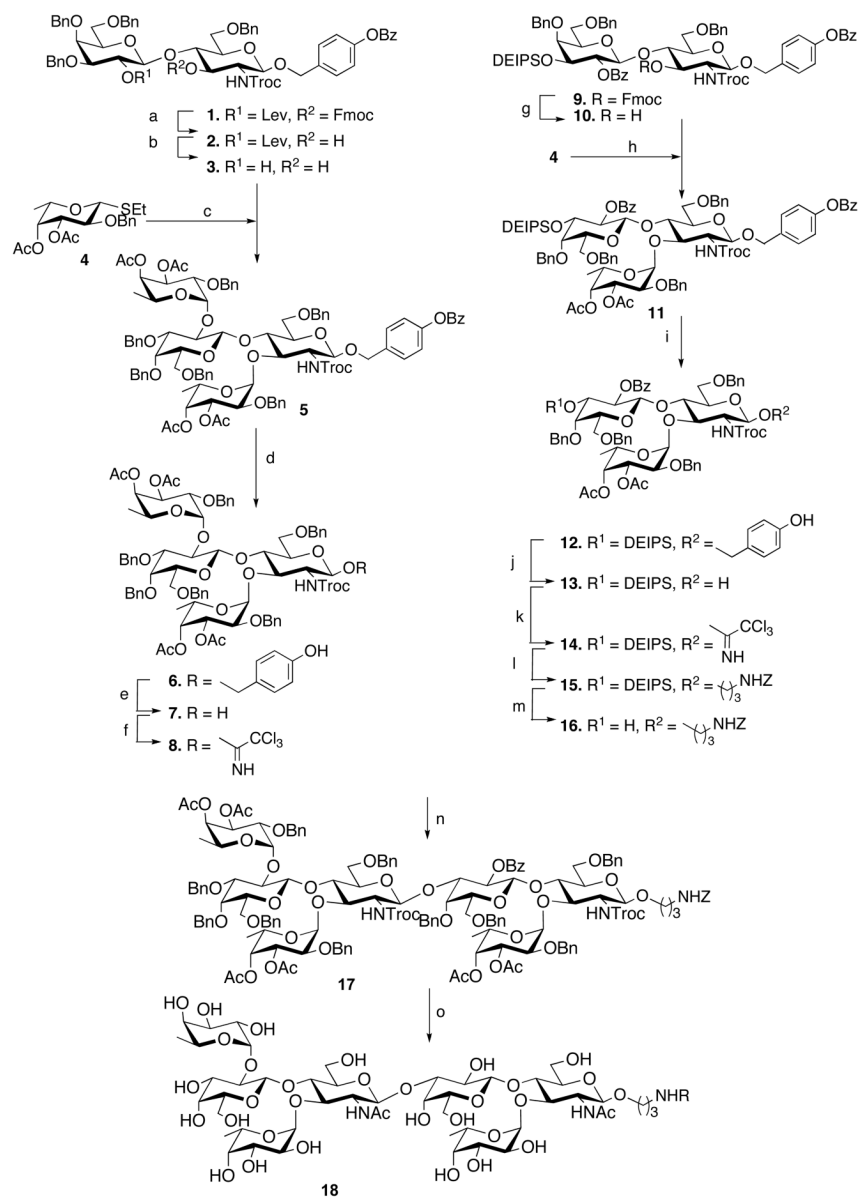
Figure 10.
Chemical structures of synthetic antigens.

Scheme 1.
Schematic presentation of the mechanism of Native Chemical Ligation (NCL).

Scheme 2.
Synthesis of the Lewis Y- Lewis X dimer. a) DCM/Et$_3$N (5/1, *v/v*) 95%; b) NH$_2$NH$_2$-HOAc, MeOH, DCM, 87%; c) NIS, TESOTf, DCM, 0 C; d) H$_2$O$_2$, Et$_3$N, THF, 82%; e) DDQ, DCM/H$_2$O 95/5, 78%; f) CCl$_3$CN, DBU, DCM, 91%; g) DCM/Et$_3$N (5/1, *v/v*), 95%; h) NIS, TESOTf, DCM, 0 C, 74%; i) H$_2$O$_2$, Et$_3$N, THF, 80%; j) DDQ, DCM/H$_2$O (95/5, *v/v*), 81%; k) CCl$_3$CN, DBU, DCM, 90%; l) BF$_3$-Et$_2$O, DCM, 86%; m) TBAF, HOAc, THF, 82%; n) NIS, TBSOTf, DCM, −30 C, 62%; o) 1: Zn, HOAc; 2: Ac$_2$O, pyridine; 3: Pd(OAc)$_2$, H$_2$, HOAc/EtOH (1/5 *v/v*) 4: NaOMe, MeOH, pH 10, 52% over four steps.
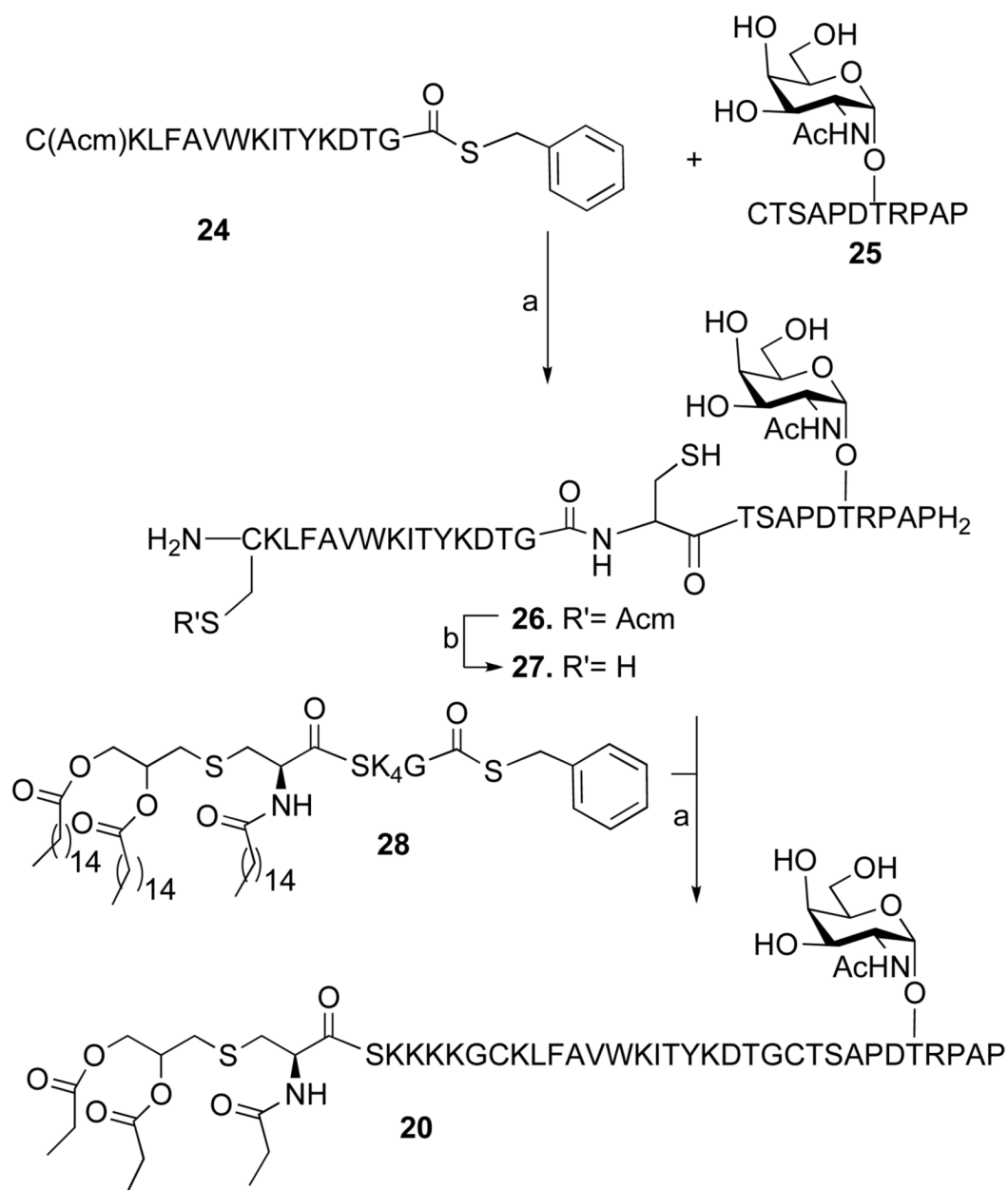
Scheme 3.
Synthesis is a tricomponent anti-cancer vaccine. a) DPC, sodium phosphate buffer (200mM) pH 7.5, TCEP (2% w/v), EDTA (0.1% w/v), MESNa (2% w/v); b) Hg(OAc)$_2$, 10% aq. HOAc, DTT.
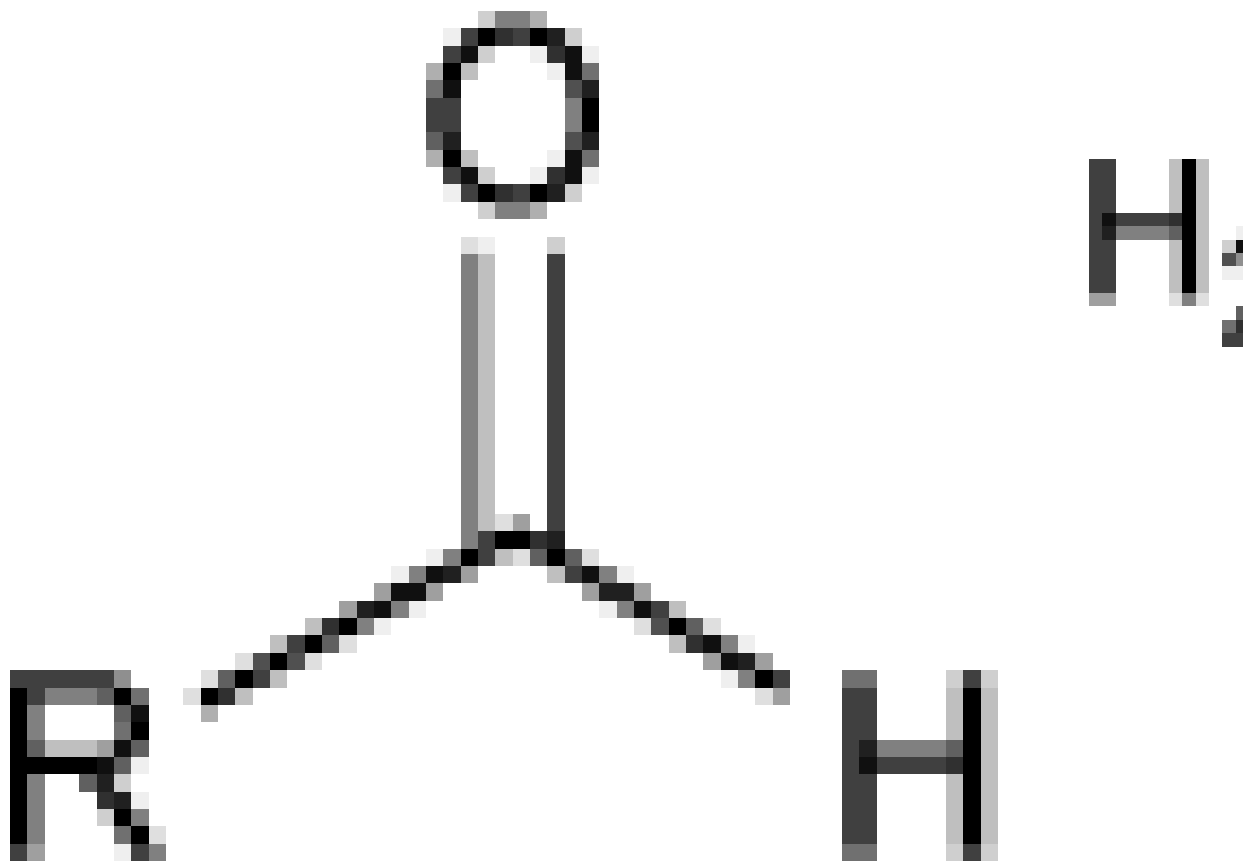
Table 1

Conjugation chemistry for ligating a peptide epitope and an oligosaccharide (or glycopeptide).

Reaction	Functional group 1
Thioalkylation	R-SH
Thiol addition	R-SH
Disulfide formation	R-SH

Reaction	Functional group 1
Oxime formation	

Reaction	Functional group 1
Hydrazone formation	![aldehyde R-CHO]
Huisgen cyclo-addition Triazole formation	$R\text{-}N_3$

Table 2

ELISA anti-MUC1 antibody titers[*] after 4 immunizations.

Immunization	Total IgG[**]	IgG1	IgG2a	IgG2b	IgG3	IgM
Pam_2CysSK_4 vaccine **19**	20,900	66,900	700	900	7,300	1,400
Pam_2CysSK_4 vaccine **19** + QS-21	30,200	113,100	23,000	6,600	17,800	1,100
Pam_3CysSK_4 vaccine **20**	169,600	389,300	56,500	42,700	116,800	7,200
Pam_3CysSK_4 vaccine **20** + QS-21	322,800	371,300	378,900	56,800	263,500	5,000

[*]
Anti-MUC1 antibody titers are presented as the median for groups of five mice. ELISA plates were coated with BSA-BrAc-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution vs. absorbance. Titers are defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

[**]
A statistical significant difference (P < 0.05) was observed between **19** *vs.* **20**.