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Perspective

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A Vision for Vaccines Built from Fully Synthetic Tumor-Associated Antigens: From the Laboratory to the Clinic

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ABSTRACT: Cancer cells may be distinguished from normal cells by cell surface displays of aberrant levels and types of carbohydrate domains. Accordingly, these tumor-associated carbohydrate antigens (TACAs) represent promising target structures for the design of anticancer vaccines. Over the past 20 years, our laboratory has sought to use the tools of chemical synthesis to develop TACA-based anticancer vaccine candidates. We provide herein a personal accounting of our laboratory's progress toward the long-standing goal of developing clinically viable fully synthetic carbohydrate-based anticancer vaccines.

INTRODUCTION

While not fully insulated from scattered skeptics and even naysayers, it is widely believed that vaccinology has had a profoundly positive effect on human health. No doubt the major triumphs of vaccine-induced immunity have been in the area of infectious diseases engendered by various bacterial, viral, and fungal pathogens. Classical vaccines have been particularly effective in targeting pathogens whose antigens are, structurally, rather stable over a considerable period of time.

Traditionally, in the mindset of academic and pharma scientists, there has been a sharp divide in the methods used to discover and evaluate small molecule drugs, as opposed to vaccines. On the whole, drugs would arise from chemical synthesis, often informed by adventitious lead structures originating from medicinal sample collections or from small molecule natural products (SMNPs). By contrast, anti-infective vaccines were fashioned by appropriate bioprocessing of whole organisms (modified to attenuate pathogenicity) or truncated to produce antigen presenting, but nonpathogenic, motifs.

By contrast, confidence that vaccinology can play an important role in oncology is far less widespread. Indeed, as we first began the project described in this retrospective, there were sound reasons, documented below in some detail, for considerable pessimism. Even before spelling out these concerns in sobering detail, suffice it to say for the moment that the hurdles that vaccines must face in infectious disease and cancer are strikingly different. In the infectious disease space, immunity must ward off or dissipate an incipient infection. In the cancer theater, a vaccine will be called upon to summon an already embattled immune system, in order to reverse, or at least contain an invasion which has already taken hold.

That we, as chemists, even began to think about such an undertaking arose from discussions with two of our colleagues at MSKCC, Drs. Philip Livingston and Kenneth Lloyd, with further encouragement from Dr. Alan Houghton and the late Dr. Lloyd Old. Through these "agenda free" exchanges, we learned about the curious tendency of various tumors to express, on their cell surfaces, carbohydrate patterns which differed from those of nonafflicted cells. Moreover, there seemed to be a relationship between tumor type and expression levels of tumor-associated carbohydrate antigens (TACAs). More detail will be provided regarding this phenomenon as our perspective unfolds. For the moment, we need only point out that, as organic chemists with a particular interest in synthesis, this information served to incite a vision which, in retrospect, seems naïvely simplistic. The TACA structures that our colleagues were showing us were, particularly by the standards of the time, quite complex and chemically challenging. The possibility of vaccinating with whole (even modified) live cancer cells was not "on the table" at the time for many obvious reasons. The chances of detaching the TACAs from the tumor stricken cells, retrieving them intact, separating them from everything else (including the whole panoply of non-TACA oligosaccharides) was, and remains, forbidding. Accordingly, we wondered whether the wisdom of organic chemistry, particularly organic synthesis, could be marshalled (and augmented) to bring the TACAs into being as homogeneous viable entities, fashionable into TACA-directed vaccines. We came to envision the emergence of fully synthetic, "vaccinizable" TACAs in much the same spirit as pertains to synthetic "small molecule" and natural product inspired drugs with which we are all accustomed. Adding to our sense of excitement was the realization that the organic chemistry needed to synthesize the TACA at that time, while substantial, would have to be considerably augmented with new methods, leading to generally useful new strategies. Many chemistry-centered questions would have to be asked and answered for this project to have any chance of success. Also not lost upon us, even from the outset, was that building the TACA "in house" would offer opportunities for creative design in fashioning the actual vaccines. In short, we came to fantasize that with sufficient imagination, diligence, and perseverance, we could synthesize the TACA and generate increasingly optimal vaccines eventually worthy of clinical evaluation in needful humans. We offer this report in the combined context of a personal retrospective and a review of the field. Happily, we were not alone in these types of dreams. As our work was progressing, it benefitted greatly from happenings in other laboratories (sometimes competitive!) around the globe. Before

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launching into the chemistry phase of our program, it is good for the reader to gain a greater appreciation of the risk parameters associated with creation of a prospective anticancer vaccine, as the project initiated and as it unfolded over time. Toward this end, we momentarily digress and recount various teachings from the field of immunology most relevant to our mission.

BACKGROUND

Tumor Immunology. A goal of contemporary cancer immunology is to delay or prevent tumor relapse or metastasis by priming the immune system to recognize and eradicate any malignant cell regrowth following removal of the primary tumor by surgery, radiation, or chemotherapy.[1] Success in this arena would require mastery of a number of unique challenges. First, strategies must be identified by which to differentiate tumor cells from normal cells at the chemical level, so that a robust and selective immune response can be evoked. Failure to solve this problem could lead to the ravages of autoimmunity of various forms.

At the clinical level, one must further contend with the fact that cancer patients are often of more advanced age and present with weakened immune systems as compared with the broader population. Moreover, there is growing evidence that tumors are capable of evading immune detection, both through immunosuppressive mechanisms and through shedding of recognition molecules that would elicit an immune response. Despite the undeniable challenges, the enormous potential benefit that would attend the development of effective anticancer vaccines has inspired a great deal of interest in the field, and the past two decades have witnessed significant progress toward this important goal.

The discoveries that tumor cells are considerably differentiated from normal cells through aberrant patterns of cell surface glycosylation[2] raised the tantalizing possibility that a TACA, when properly presented to the human immune system, might evoke an adaptive immune response, culminating in the selective eradication of tumor cells displaying the epitopes of interest. TACAs represent a particularly promising class of potential anticancer epitope, as they are among the most prevalent antigens detected on cancer cell surfaces.[3,4] Moreover, certain TACA structures are commonly overexpressed across a number of different tumor types. Accordingly, it might be possible to develop broadly useful vaccine constructs which could be used in the treatment of a range of cancer types. Intriguingly, a natural TACA-directed antibody response to the tumor state has been observed in a small minority of cancer patients; the presence of anti-TACA antibodies in these patients is correlated with significant improvement in survival rates.[5]

As intriguing as is the central idea, a number of sobering fundamental challenges would have to be addressed en route to the development of a viable TACA-based anticancer vaccine.[6,7] First, many tumor-associated carbohydrates are also present (to some degree) on normal cell surfaces and are thus auto- or self-antigens. Moreover, there is a significant level of microheterogeneity in tumor cell carbohydrate expression.[3] In fact, a single tumor cell might overexpress an array of different TACAs over the course of its lifetime. Thus, even if chemistry could meet the challenge of producing structurally homogeneous TACAs, clinical success was far from certain.

It should also be understood that the central idea of inducing a TACA-based immune response in cancer is itself fraught with uncertainty. Most carbohydrate antigens are strictly B-cell epitopes and, in the absence of external mediators, typically induce a weak, T-cell-independent humoral response. Thus, as shown in Figure 1a, carbohydrate antigens activate B cells

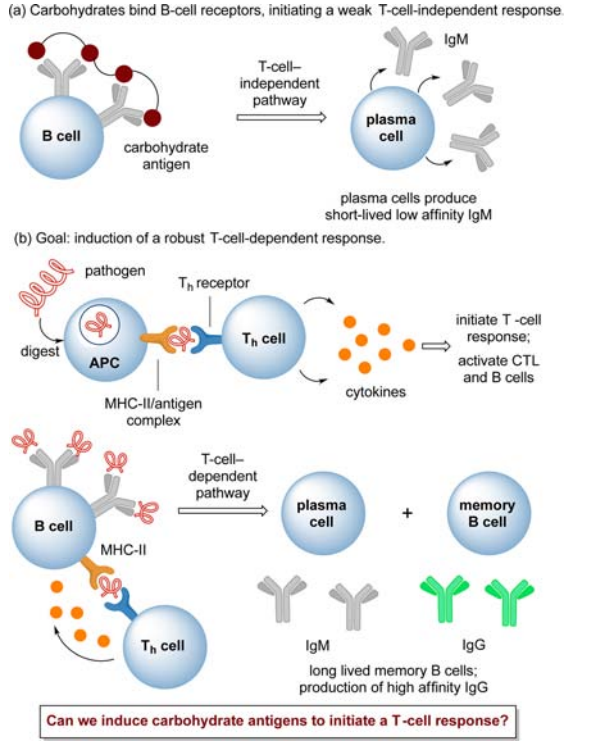

Figure 1. Immunological response to carbohydrate antigens.

through cross-linking of surface B-cell receptors. These partially activated B cells differentiate to plasma B cells, which produce only short-lived, low-affinity IgM antibodies. Induction of the types of robust, long-lived immune responses required for effective cancer immunotherapy necessitates full activation of these antigen-bound B cells and is achieved only through initiation of a T-cell-dependent pathway.

The sequence of events that culminates in induction of a T-cell-dependent immunogenic response is adumbrated in Figure 1b. An antigen-presenting cell (APC) engulfs and lyses the foreign body (or pathogen) and presents antigenic portions of the invading body on its cell surface, through a linkage to the major histocompatibility (MHC-II) protein. Helper T (T_h) cells then bind to the MHC-II/antigen complex through the T-cell receptor. This interaction triggers the release of cytokines and chemokines from the T_h cell, leading to the activation of both cytotoxic T cells (CTLs) and B cells. As shown in Figure 1b, the crucial association of activated T_h cells with antigen-bound B cells (bearing the same epitopes) leads to the release of powerful cytokines that serve to fully activate the B cells, resulting in class switching and affinity maturation to produce high-affinity IgG antibodies and, most importantly, long-lived memory B-cells. Because the formation of memory B cells is seen as central to the induction of a robust and long-lasting immunologic response, a fundamental goal of tumor immunology is to induce T-cell-dependent responses that culminate in the formation of memory B cells. However, aside from a few notable exceptions,[8] carbohydrates cannot induce a T-cell response on their own because they are not presented by the MHC-II complex on APCs. In the absence of MHC-II presentation

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to the T_h cell, a T-cell-dependent response presumably cannot be evoked. A solution to this challenge could entail installation of immunoenhancing element onto the vaccine construct. This arm of the vaccine could promote induction of a potent T-cell response.

These daunting constraints not withstanding, we pressed forward hoping to learn as we went along. Figure 2 outlines a

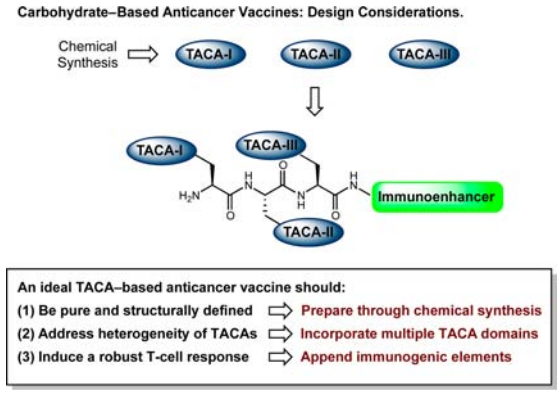

Figure 2. Design of synthetic TACA-based anticancer vaccines.

template for the design elements that have guided our own efforts toward the development of clinically viable carbohydrate-based anticancer vaccines. First, the carbohydrate domains of the vaccine construct must be pure and structurally defined. This requirement is fulfilled through recourse to de novo synthetic methods. We note, in passing, that clinical approval of a fully synthetic, homogeneous carbohydrate-based vaccine construct would represent an important milestone in medicinal chemistry. To our knowledge, the *Haemophilus influenza* Type B (Hib) vaccine (consisting of oligomers of ribosylribitol-phosphate) is the only synthetic carbohydrate-based vaccine approved for any type of clinical use.[9]

Next, to address the microheterogeneity of TACA expression on tumor cell surfaces, the vaccine construct should eventually incorporate multiple varied TACA domains associated with a single cancer type. Finally, the goal of inducing a robust T-cell response can be met by appending the TACA-based construct to known immunogenic elements, such as carrier proteins. Carrier proteins are potent immunoenhancers, incorporating peptide sequences that are substrates for the MHC-II complex of APCs (Figure 1b). Covalent linkage of B-cell antigens (TACAs) to carrier proteins promotes initiation of T-cell-dependent pathways and the formation of IgG antibodies against the B-cell epitopes. Co-administration of a nonspecific immunoadjuvant (such as QS21) serves to further potentiate the immune response.[10,11]

With the basis for our own hopes and concerns now delineated, we provide below a synopsis of our laboratory's 20-year program directed toward the long-term objective of developing a clinically effective TACA-based anticancer vaccine.[12] As noted earlier, a number of other groups have registered impressive advances in the field of synthetic TACA-based tumor immunology. Accordingly, though the purpose of this account is to convey the trajectory of our own path toward our goal, relevant highlights from other laboratories are interspersed.

TACA Classes. Figure 3 depicts representative members of the four main classes of TACAs. Glycans of the Globo class, including Globo-H, Gb5, and Gb3, are overexpressed as glycolipids on the surfaces of a range of tumors, including those of the breast, colon, lung, ovaries, and prostate. Interestingly, the Wong group recently found that Gb5 is widely overexpressed on breast cancer stem cells, a subpopulation of cells that are capable of self-renewal and differentiation.[13] Because cancer stem cells play an important role in mediating tumor regrowth and metastasis,[14] the Gb5 pentasaccharide is considered a particularly attractive antigen for the treatment of breast cancer. The blood group determinants are a class of Lewis antigens overexpressed as glycosphingolipids in a range of tumors. The gangliosides, including GM2, GM3, GD2, GD3, and fucosyl-GM1, are linked to cell surface lipids and overexpressed in melanomas and in lung, colon, renal, and prostate cancers. Finally, the mucin-related TACAs (Tn, TF, STn) are attached through α-O-Ser/Thr linkages to membrane-bound mucin proteins and are implicated in epithelial cancers. Using strategies developed in our laboratory, we have synthesized (often for the first time) representative members of each of these four main TACA classes.

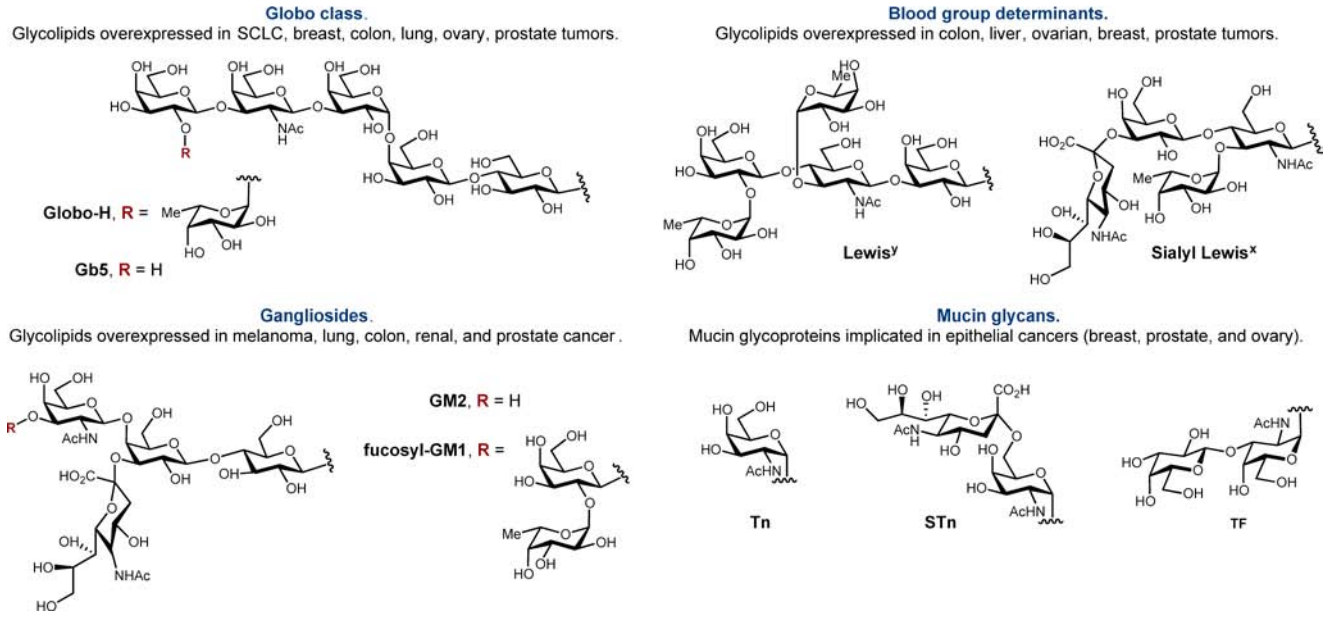

Figure 3. Representative members of the major TACA classes.

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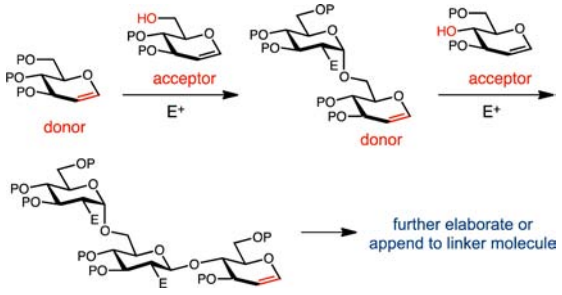
Glycal Assembly. Our entry into the field of carbohydrate-based anticancer vaccines arose from an initiative to develop improved methods for the synthesis of complex oligosaccharides. Earlier, and by a thought process delineated elsewhere,[15] we conceived of the paradigm of "glycal assembly", whereby individual glycal units, possessing three hydroxyl groups and an olefinic handle, are iteratively merged in a controlled fashion to build complex carbohydrate sectors. We were not the first to recognize that a glycal can, on suitable activation, give rise to a glycosyl donor. That said, our laboratory did provide the first demonstration that such glycals, bearing at least one hydroxyl group, could also serve as viable glycosyl acceptors, as shown in Scheme 1. The product that arises from glycal assembly

Scheme 1. Glycal Assembly Approach to Carbohydrate Synthesis[a]

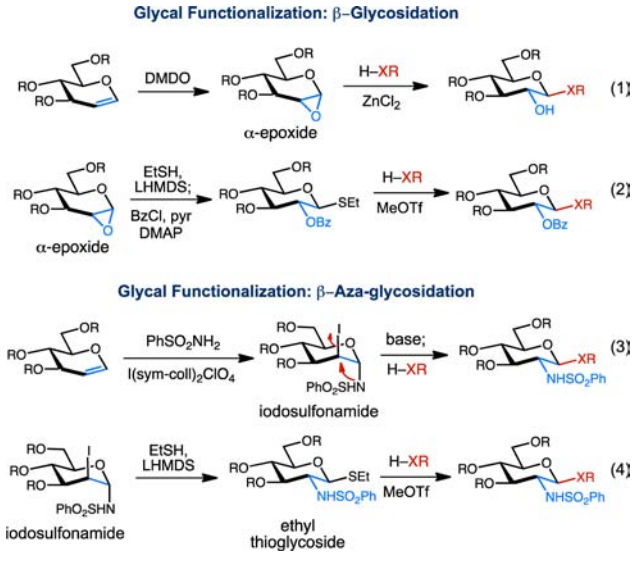


[a]Adapted from ref 12e, copyright 2010, Wiley.

provides still another glycal for subsequent iteration. With the reiterations complete, the terminal glycal linkage of the last acceptor provides a linkage opportunity to other biodomains.

Methods for the functionalization of terminal glycal motifs (largely developed in our group) are presented in Scheme 2.

Scheme 2. Methods for Glycal Functionalization[a]



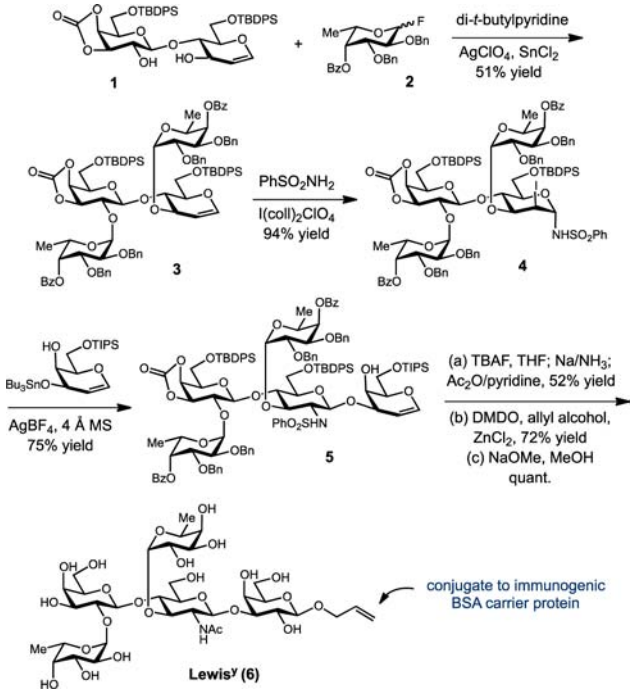
[a]Adapted from ref 12e, copyright 2010, Wiley.

Stereoselective functionalization may proceed through the intermediacy of an α-epoxide species (Scheme 2, eqs 1 and 2) or an iodosulfonamide, which is converted to a sulfonylaziridine upon exposure to base (eqs 3 and 4). In straightforward cases, the α-epoxides or sulfonylaziridines (arising from the corresponding iodosulfonamide) are susceptible to direct nucleophilic addition by H–XR (eqs 1 and 3). In some substrates, however, the steric demands of the system require recourse to a two-stage process, involving the formation of an intermediate ethyl thioglycoside species (eqs 2 and 4). These compounds are then subjected to nucleophilic displacement, as shown.

Synthesis and Evaluation of Monomeric Vaccine Conjugates. Our earliest cancer vaccine-based research efforts were directed toward the synthesis of a series of monomeric TACA-based vaccine conjugates, in which a single TACA is conjugated to an immunogenic carrier protein, such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). We describe below the synthesis and immunological evaluation of several representative monomeric vaccine constructs.

Lewis^y–KLH. We began our program with the synthesis of the blood group determinant Lewis^y and its conjugation to KLH carrier protein. The synthesis of the Lewis^y pentasaccharide was emblematic of the emerging glycal assembly and functionalization logic outlined in Schemes 1 and 2 above.[16] It

Scheme 3. Synthesis of Lewis^y 6[a]



[a]Adapted from ref 12a, copyright 2000, Wiley.

is rather pleasingly concise. In brief, disaccharide **1**, bearing free hydroxyl groups at C_3 and $C_{2'}$, was accessed from commercially available lactose. Bis-fucosylation of acceptor **1** with fluorinated donor **2** afforded the tetrasaccharide adduct, **3**, in 51% yield. The glycal motif was next converted to the corresponding iodosulfonamide donor, **4**, and subsequent azaglycosylation with the 3-stannyl ether of galactal in the presence of $AgBF_4$ gave rise to the pentasaccharide glycal, **5**, possessing the requisite stereochemical disposition. The latter was converted to the Lewis^y target compound **6** through sequential global deprotection, peracetylation, α-epoxidation, epoxide opening with allyl alcohol, and removal of the ester protecting groups. The allylic motif provided a convenient functional handle for conjugation to the immunogenic BSA and KLH carrier proteins.

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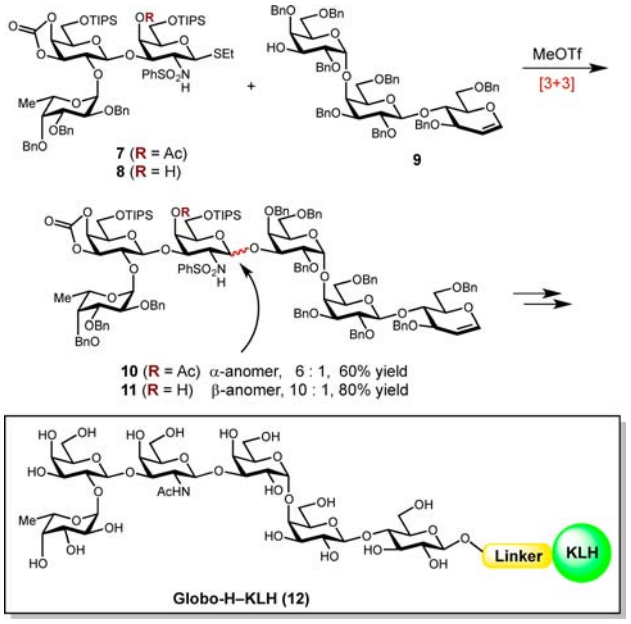
Of the Lewis^y vaccine constructs synthesized, the Lewis^y–KLH conjugate was found to be most effective in preclinical immunological assays. Mice inoculated with Lewis^y–KLH, coadministered with QS-21 immunoadjuvant, were observed to generate IgM and, to a lesser extent, IgG antibodies. The antibodies thus formed were found to strongly and selectively bind Lewis^y-positive MCF-7 cell lines. Cytotoxicity tests for antibody-dependent complement-mediated lysis revealed these antibodies to be selectively toxic to Lewis^y positive cell lines.[17]

On the basis of these findings, Lewis^y was advanced to phase I clinical trials against ovarian cancer at MSKCC. Although an immune response to the Lewis^y antigen was observed in clinical settings, the majority of the antibodies formed were of the low-affinity IgM type.[18] The vaccine has not yet been advanced to phase II evaluation.

Globo-H–KLH. The Globo-H hexasaccharide was first isolated from the human breast cancer cell line, MCF-7, and subsequently found to be implicated in a range of different cancer types, including colon, lung, prostate, and ovarian.[19] In 1995, we disclosed the first total synthesis of Globo-H, through a route designed to maximize synthetic convergence.[20] The ABC (**9**) and DEF (**7**) trisaccharides were synthesized through glycal assembly techniques. We then sought to accomplish the [3 + 3] coupling of the component trisaccharide domains. In the event, thioglycoside **7** and glycal **9** underwent methyl triflate-promoted coupling to afford a hexasaccharide product, **10**, as a 6:1 ratio of stereoisomers at the C–D ring junction (Scheme 4). However, further structural evaluation revealed the

Scheme 4. Synthesis of Globo-H–KLH[a]



[a]Adapted from ref 12d, copyright 2007, American Chemical Society.

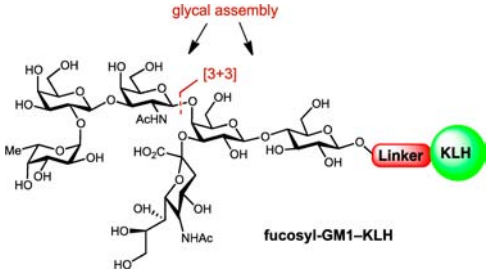
major product to possess the undesired α-anomeric disposition. We hypothesized that this unexpected product distribution might be a consequence of the failure of the sulfonamide of **7** to participate in activation of the donor. We reasoned that perhaps certain structural features of **7** precluded formation of the requisite cyclic sulfonamide (which would have dictated glycosidation from the desired β-face) and the coupling instead proceeded through the intermediacy of an onium species. If this hypothesis were correct, then we might be able to bias the reaction toward formation of the cyclic sulfonamide intermediate through minor perturbations of the donor structure. Toward this end, we synthesized a slightly modified DEF domain, **8**, in which a free hydroxyl replaces the acetate group at C_4 of the future D ring. As shown in Scheme 4, methyl triflate-mediated coupling of **8** with **9** afforded the requisite β-anomeric hexasaccharide adduct, **11**, in 80% yield and 10:1 dr. This result serves to highlight the extraordinary sensitivity of these types of coupling reactions to small perturbations on the donor ring.

In the ensuing years, our laboratory developed a second-generation synthetic strategy, which provides more ready access to significant quantities of material.[21] Other groups have also disclosed efficient synthetic routes toward the Globo-H glycan.[22]

Preclinical findings obtained with our synthetic Globo-H–KLH conjugate were very encouraging.[23] Mice immunized with the vaccine conjugate produced high-titer IgM and IgG antibody responses to Globo-H. Moreover, these antibodies were unreactive against Globo-H-negative B78.2 cells but were capable of effectively inducing complement-mediated lysis against Globo-H-positive MCF-7 cell lines.

On the basis of these promising preclinical data, the Globo-H–KLH vaccine was advanced to phase I clinical trials at MSKCC against prostate cancer[24] and, subsequently, against breast cancer.[25] Both studies established the safety and baseline immunogenicity of the vaccine across a range of cancer stages. In the prostate cancer study, patients uniformly exhibited strong IgM antibody responses. The antibodies thus generated were selectively reactive against Globo-H-positive cell lines, as evidenced by flow cytometry and complement-mediated lysis studies. The Globo-H–KLH vaccine conjugate has since progressed to phase II/III clinical trials against breast cancer, both in the United States and abroad. Definitive reports on the results must await unblinding of the data and further analysis.

Fucosyl-GM1–KLH. The fucosyl-GM1 hexasaccharide is not present in normal lung or tissue cells but is abundantly overexpressed on many small-cell lung cancer (SCLC) tumors. Accordingly, this ganglioside is considered a promising target for tumor immunotherapy in SCLC patients. In 2004, we accomplished the synthesis of fucosyl-GM1 through application of the glycal assembly and coupling strategies described above.[26] Conjugation to carrier protein through a linker molecule yielded the fully synthetic fucosyl-GM1-KLH vaccine candidate shown below. The immunogenicity of this construct was evaluated in patients with SCLC in a phase I clinical trial conducted at MSKCC. This study revealed the ability of the fucosyl-GM1-KLH construct to elicit antibodies, mainly of the IgM type, against the oligosaccharide component. Moreover, sera samples isolated from responsive patients were typically capable of inducing complement-mediated cytotoxicity of fucosyl-GM1-positive tumor cells.



Monovalent Clustered Vaccines. Our first-generation efforts culminated in the syntheses and clinical evaluations of promising monovalent vaccine candidates, including

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Globo-H-KLH, Lewis^y-KLH, and fucosyl-GM1-KLH. We next sought to develop increasingly sophisticated constructs that would more closely resemble the architectures typically encountered on tumor cell surfaces. Along these lines, it is well-known that the tumor-associated mucin-bound glycophorins, Tn, TF, STn, are presented on adjacent Ser and Thr residues in repeating clusters of three to five saccharides. Such carbohydrate clusters appear to be the preferred targets of monoclonal antibodies. With the goal of eliciting antibodies that would most effectively target epithelial tumor cells, we synthesized a series of clustered vaccine constructs, wherein Tn, TF, or STn antigens are presented in triplicate on adjacent Ser or Thr amino acid residues (Tn(c), TF(c), and STn(c), Figure 4).[27]

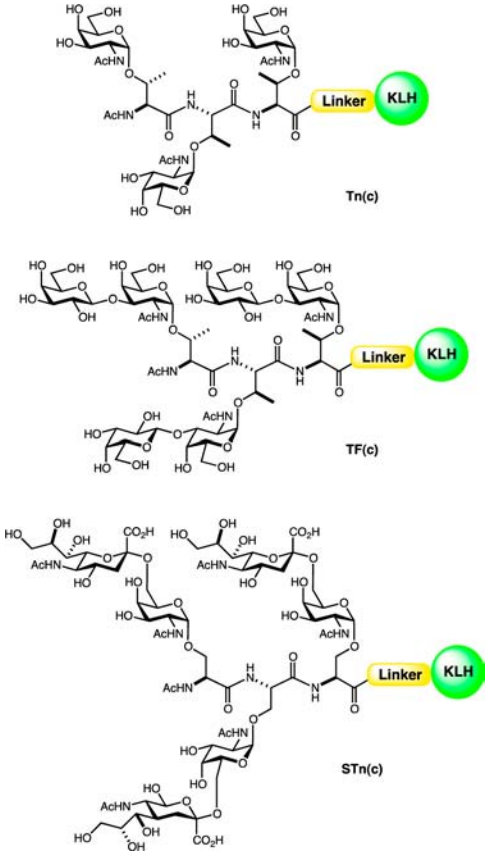

Figure 4. Clustered mucin glycopeptides.

As hoped, these clustered vaccines were generally observed to exhibit enhanced levels of antibody production compared to their monomeric analogs. Several of these compounds have been evaluated in clinical trials.[28]

Strategies for Addressing Tumor Microheterogeneity. Despite their potential clinical utility, our first- and second-generation monomeric and clustered vaccine candidates did not address the phenomenon of tumor microheterogeneity discussed earlier. While the advancement of Globo-H toward possible registration is still underway, we have undertaken "second-generation" strategies which confront the microheterogeneity problem. The ultimate approach we preferred was to incorporate a series of TACAs into a fully synthetic unimolecular construct. While the chemistry to fashion such a unique vaccine was being crafted, we first turned to the "pooled monomeric" vaccine strategy envisioned by our collaborator, Dr. Philip Livingston.

Pooled Monomeric Vaccines. From a synthetic perspective, implementation of the pooled monomeric approach is very straightforward. Mixtures of monomeric TACA−KLH conjugates are simply pooled together and injected simultaneously, with the aim of eliciting an immune response against each of the component antigens. In one representative preclinical study, depicted below, mice immunized with seven different TACA−KLH conjugates generated antibodies against each antigen except GM2 (Figure 5). Moreover, the antibodies thus

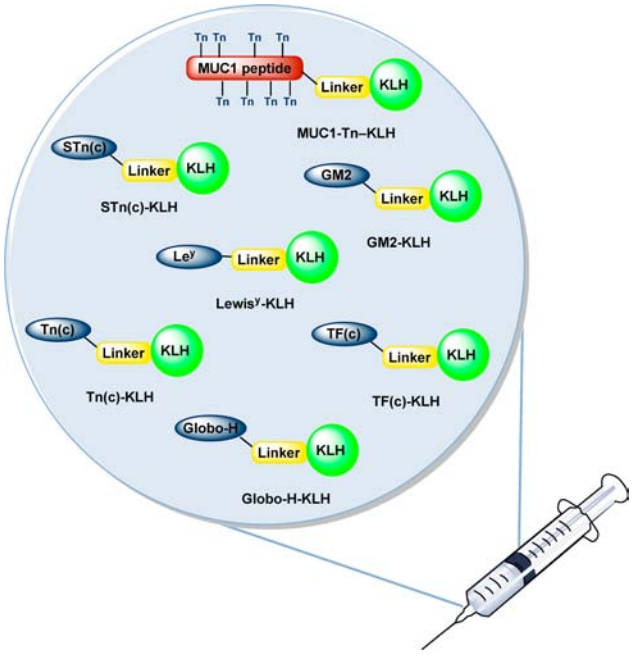

Figure 5. Pooled monomeric vaccine strategy.

generated were generally of both the IgM and IgG types, with the exception of Lewis^y, which elicited only IgM antibodies.[29]

This vaccine strategy was further evaluated in phase I clinical trials.[30] The pooled heptavalent mixture was co-administered with QS21 to patients in remission with ovarian, fallopian tube, or peritoneal cancer, and serologic responses were monitored. Eight of the nine patients generated antibodies against at least three of the component antigens. Disappointingly, however, the median antibody titers for all antigens were significantly reduced in comparison to the levels achieved through vaccination with the corresponding single antigen conjugates. It is postulated that the reduced serological responses observed in this study are attributable to the large quantities of KLH required under this strategy. In fact, it has been shown that excess levels of carrier protein may precipitate a decreased immunogenic response to the carbohydrate antigen.

Unimolecular Multiantigenic Vaccines. Our laboratory has more recently begun to pursue the more clinically pleasing and operationally sensible approach, involving the design of unimolecular multiantigenic vaccine constructs. According to the strategy highlighted in Figure 6, multiple synthetically derived TACAs associated with a particular cancer type are joined on a single peptide backbone and conjugated to the carrier protein. This strategy has a number of important advantages over alternative approaches to TACA vaccines. First, the inclusion of multiple TACAs will hopefully effectively address the issue of heterogeneity of tumor cell carbohydrate expression. Moreover, it must be acknowledged that merger of the TACA domain with

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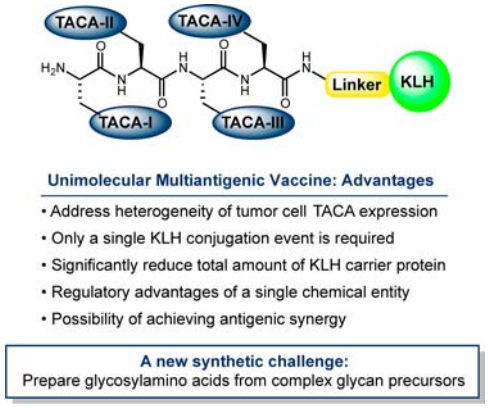


Figure 6. Unimolecular multiantigenic vaccine strategy.

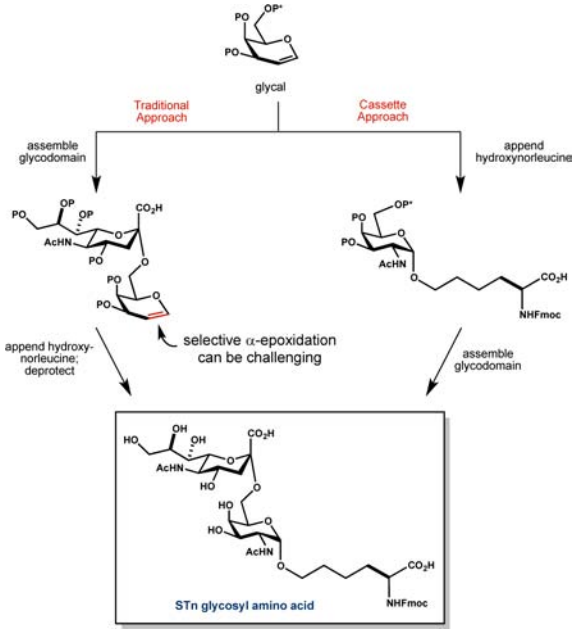
KLH carrier protein presents a significant operational challenge, and such conjugation reactions typically proceed with low overall yield. Under the unimolecular strategy, a single conjugation event serves to append the fully elaborated antigenic glycopeptide to the carrier protein. This design feature offers the further advantage that significantly reduced quantities of KLH are required, in comparison with the pooled monomeric vaccine strategy. As noted above, use of excessive amounts of KLH can lead to suppression of the immune response to the TACA. We further take note of the regulatory advantages associated with registration of a single chemical entity. Finally, it is conceivable that antigen synergy could be achieved through the installation of multiple different TACAs upon a single molecular scaffold.

With these considerations in mind, we aimed to synthesize a series of unimolecular constructs for evaluation in preclinical and clinical settings. However, before commencing with this effort, we would need to meet a new synthetic challenge. Where our monomeric vaccine constructs had consisted of a carbohydrate domain linked to immunogenic carrier protein, assembly of the proposed glycopeptide-based unimolecular multiantigenic vaccines would require us to develop means by which to efficiently convert complex glycan precursors to the corresponding glycosylamino acids. These would then be iteratively linked en route to the glycopeptide backbone. This new synthetic challenge propelled us to develop a new approach to the preparation of glycosylamino acids, which we term "cassette assembly".

CASSETTE ASSEMBLY

A Modified Approach to Glycosylamino Acid Synthesis. Traditional approaches to glycosylamino acid synthesis commence with preparation of the carbohydrate through glycal assembly methods. Subjection of the terminal glycal of the fully protected glycan domain to α-epoxidation followed by further functionalization culminates in the installation of the amino acid moiety (Scheme 5). However, it is often difficult to achieve adequate levels of α-selective epoxidation of the fully elaborated glycal precursor. Accordingly, we have developed an alternative strategy en route to complex glycosylamino acids, termed the cassette assembly approach.[31] As outlined in Scheme 5, this approach commences with the coupling of hydroxynorleucine amino acid with an appropriately protected monosaccharide glycal. The requisite glycodomain is then further elaborated, with the amino acid moiety already in place. Alternatively, an α-allyl functionality may be appended at the stage of the monosaccharide. Following assembly of the glycodomain, an olefin cross-metathesis/hydrogenation sequence serves to

Scheme 5. "Cassette Assembly" Approach to Glycosylamino Acids[a]



[a]Adapted from ref 12d, copyright 2007, American Chemical Society.

install the amino acid motif. Though requiring some compromise in terms of convergence, these methods offer the crucial advantage that the problematic α-epoxidation step is addressed at the outset of the synthesis, rather than in the context of the highly valuable, fully assembled oligosaccharide domain. Accordingly, we typically adopt these preferred strategies toward the assembly of complex glycosylamino acid precursors en route to unimolecular multiantigenic domains.

UNIMOLECULAR TRIVALENT VACCINE

Proof of Concept. Having established effective methods for the synthesis of TACA domains and their corresponding glycosylamino acids, we sought to synthesize and evaluate a unimolecular multiantigenic vaccine conjugate. As a proof of concept, a KLH-conjugated trivalent vaccine construct, incorporating the Globo-H, Tn, and Lewis^y antigens, was synthesized in our laboratory.[32] We were pleased to observe that mice immunized

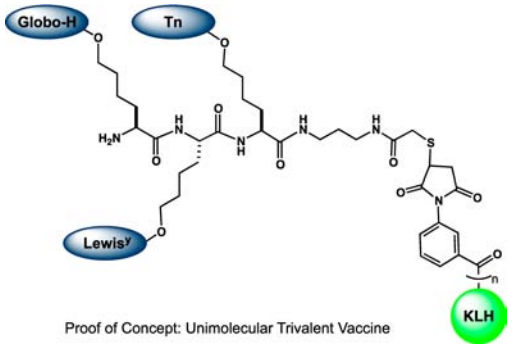


Figure 7. An immunogenic trivalent vaccine conjugate.

with this construct produced antibodies against each of the three component TACAs. Moreover, the mouse sera were shown to react strongly with the MCF-7 breast cancer cell line. We had thus demonstrated, for the first time, the feasibility of incorporating

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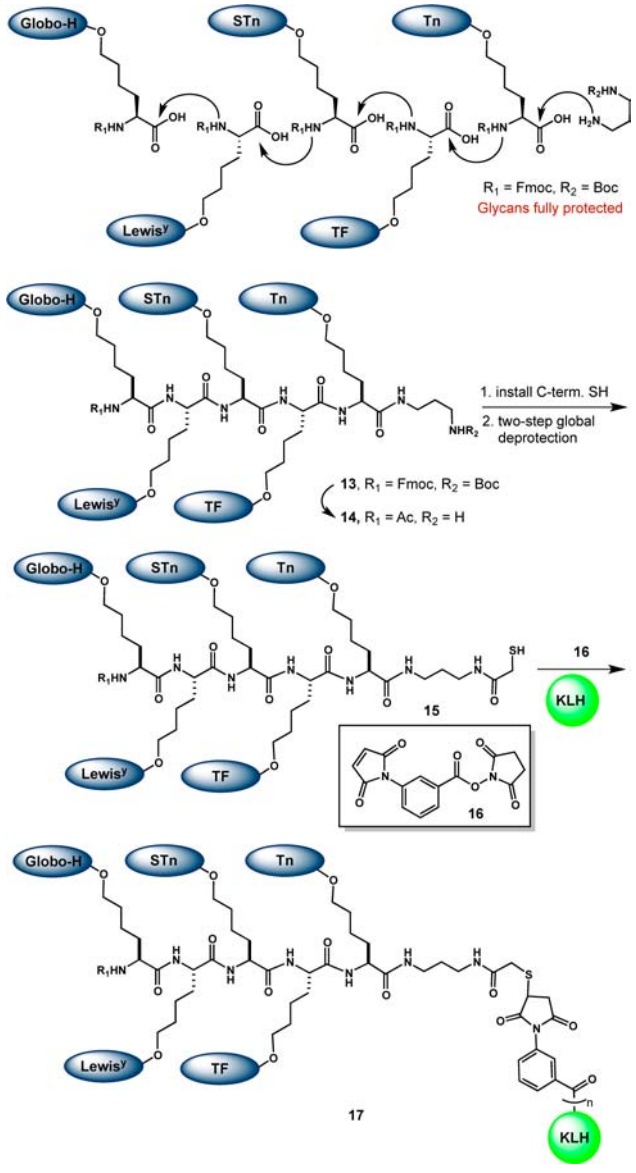
multiple different TACAs on a single peptide backbone as a viable strategy to address issues of tumor microheterogeneity.

First- and Second-Generation Unimolecular Pentavalent Vaccine Conjugates. Having established the viability of this multiantigenic paradigm, we next undertook the synthesis of a pentavalent vaccine construct bearing five different TACAs associated with breast and ovarian cancer. The synthesis of this first-generation pentavalent vaccine (**17**) is summarized in Scheme 6.[33] Thus, the component glycosylamino acids were prepared, in fully protected form, through recourse to the cassette assembly approach described above (Scheme 5). The Tn glycosylamino acid was first equipped with the *t*-butyl-*N*-(3-aminopropyl)carbamoyl group, which acts as a partial linker. Next, a series of iterative Fmoc deprotection and coupling steps served to construct the pentapeptide backbone (**13**). The latter was converted to **14** in a straightforward manner. Installation of the linker followed by global deprotection yielded **15**, which was conjugated to KLH carrier protein, derivatized with maleimide **16**. The unimolecular pentavalent vaccine construct **17** was determined to have a glycopeptide:KLH ratio of ~228:1.

Scheme 6. First-Generation Unimolecular Pentavalent Vaccine Conjugate (17)[a]



[a]Adapted from ref 12d, copyright 2007, American Chemical Society.

Preliminary preclinical studies provided further support for the viability of the unimolecular vaccine strategy. ELISA studies revealed that mice inoculated with **17** produced antibodies against four of the five component antigens; only the weakly immunogenic Lewis^y antigen failed to elicit an immune response. Furthermore, in FACS studies, the antibodies thus raised were observed to be highly reactive against three different cell lines overexpressing the target antigens.

On the basis of these encouraging findings, we proceeded to synthesize a second-generation unimolecular pentavalent construct, wherein the poorly immunogenic Lewis^y antigen would be replaced with the GM2 ganglioside (Figure 8).[34]

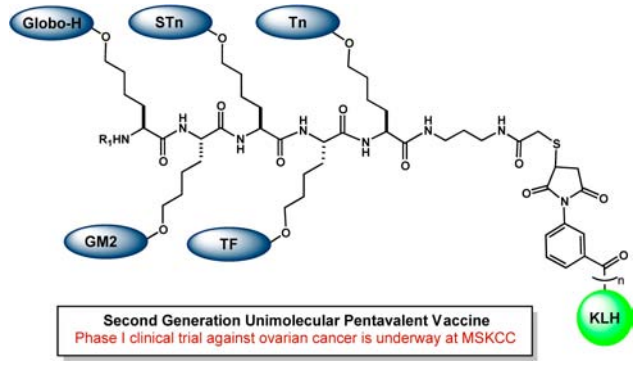


Figure 8. Second-generation unimolecular pentavalent vaccine.

Preclinical evaluations with this vaccine conjugate were very positive, indicating an antibody response against each of the five component antigens. Accordingly, this construct was advanced to a phase I clinical trial in ovarian cancer patients at MSKCC, and the study is nearing completion. Full evaluation of promising preliminary serological data must await completion of the trial.

STRATEGIES FOR AUGMENTING THE T-CELL RESPONSE

A Work in Progress. As described above, the major focus of carbohydrate-based tumor immunology has been on the identification of effective carbohydrate-based antigens and the development of strategies for the presentation of these antigens to the immune system. Most efforts have traditionally relied upon the use of a carrier protein, such as KLH, and a nonspecific immunoadjuvant, such as QS21, to enhance the T-cell response. However, more recently, increased attention has been devoted to the development of alternative approaches to augmenting T-cell-based immunogenic pathways, which either obviate or supplement standard carrier protein-based strategies. These efforts arise from a growing recognition of the challenges associated with the use of immunogenic carrier proteins. Drawbacks to the carrier protein strategy include: (1) the aforementioned difficulties associated with protein conjugation techniques, which are often low-yielding and may suffer from issues of reproducibility; and (2) the potential of the carrier protein and the linker molecule to themselves evoke strong immunogenic responses, thereby leading to a diminution of the antibody response to the TACA.

We summarize below several promising new approaches to the development of novel protein carriers as well as carrier protein-free TACA vaccines. Alternative strategies, not discussed here, include the use of dendrimers[35] or zwitterionic polysaccharides[36] as replacements for carrier protein.

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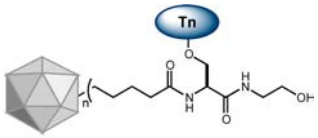
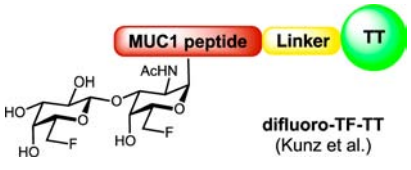
■ ALTERNATIVES TO CARRIER PROTEIN

Virus-Like Particles as Immunoenhancing Elements. Virus-like particles (VLPs), naturally occurring systems composed of subunit proteins that undergo ordered self-assembly, have recently emerged as a promising class of TACA carrier molecules. These systems are safe and not infectious to humans, yet are highly immunogenic due to a number of favorable properties, including: (1) their size, which promotes uptake by APCs; (2) their repetitive structure, which facilitates B-cell recognition; and (3) their ability to cross-link B-cell receptors. Moreover, VLPs permit the high-density and well-ordered presentation of antigens to the immune system. Finn, Huang, and co-workers have been exploring the feasibility of employing VLPs, including cowpea mosaic virus (CPMV),[37] tobacco mosaic virus (TMV),[38] and bacteriophage Qβ capsids,[39] as alternative delivery vehicles for carbohydrate antigens. In one recent preclinical study, the researchers demonstrated the ability of a Qβ–Tn conjugate to effectively induce strong anti-Tn IgG antibodies. Noting that the Tn epitope is generally presented in clustered form on tumor cell surface mucin proteins (see Figure 4), the researchers installed a high density of multiple copies of the glycan onto the Qβ scaffold, thus mimicking the native "clustered" presentation. Although the applicability of this strategy to clinical settings has yet to be established, VLPs conceivably offer an attractive new delivery tool for TACA vaccines.



Unimolecular Multicomponent Vaccines. Kunz and co-workers have pioneered the synthesis and evaluation of vaccine constructs incorporating tumor-associated mucin-derived glycopeptides.[40] Epithelial membrane-bound mucin proteins are overexpressed on tumor cell sufaces and display truncated carbohydrate domains (cf., TF, Tn, STn). The MUC1 glycopeptide, in particular, has emerged as an attractive antigen for tumor vaccine design. Early work by Kunz et al. explored "two-component" approaches where MUC1 motifs were paired with different T-cell peptide determinants in the absence of carrier.[41] Although high titers of specific antibodies could be induced in animals, the overall response rate was low. More recently, Kunz et al. have demonstrated reliable and robust immunogenicity of tumor-associated MUC1-glycopeptides conjugated to carrier proteins.[42] In one recent example, researchers prepared a difluoro-TF analog, designed with the goal of enhancing TACA biostability. When appended to MUC1 peptide and conjugated to tetanus toxoid (TT) carrier protein, the vaccine candidate was found to exhibit excellent immunogenic activity in preclinical settings.[43] Importantly, IgG antibodies elicited from this vaccine conjugate were found to recognize the native TF antigens present on the MCF-7 breast cancer cell lines. Ye et al. have observed similar effects of fluorination on derivatives of the STn antigen.[44]



A number of laboratories have pursued "multicomponent" approaches with the goal of eliminating the carrier protein and augmenting the immune response (Figure 9). Researchers in the

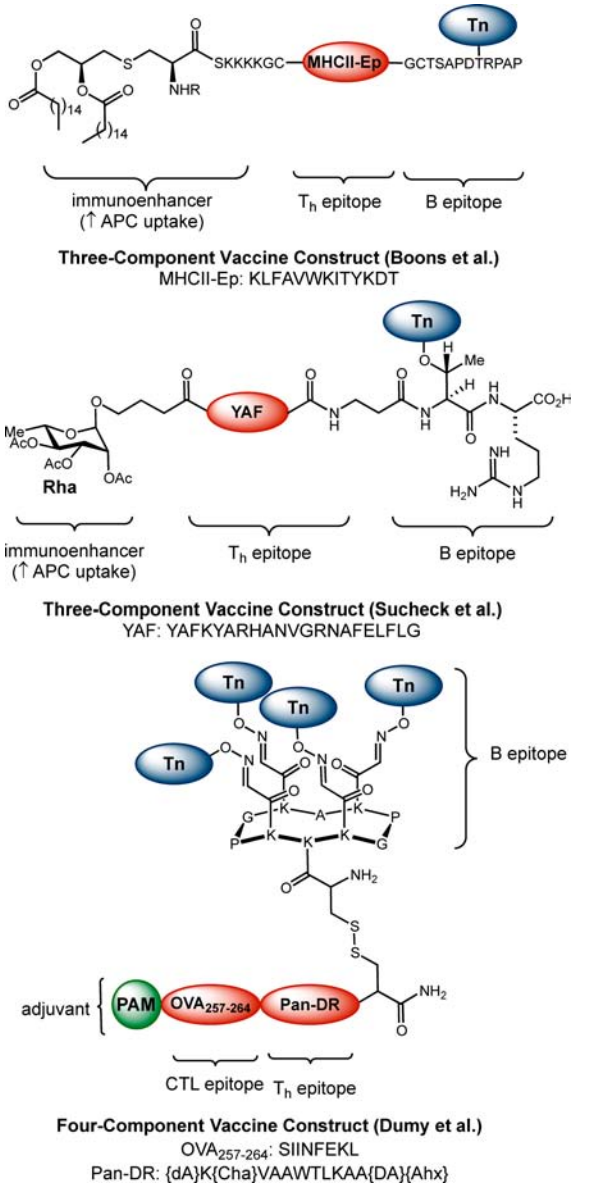


Figure 9. Multicomponent vaccine constructs: studies from the Boons, Sucheck, and Dumy laboratories.

Boons laboratory have synthesized a menu of multicomponent vaccines, designed to incorporate orthogonal immunogenic elements.[45] One impressive example is a three-component vaccine construct, incorporating: (1) the MUC1-Tn B-cell epitope, (2) a peptidyl MHCII binding sequence, which acts as a T_h epitope, and (3) the lipopeptide Pam_3CysSK_4, a Toll-like receptor 2 (TLR2) ligand that acts as an immunoenhancer by increasing APC uptake and upregulating production of cytokines and costimulatory proteins.[45c] Indeed, this vaccine elicited remarkably high levels of IgG antibodies, which effectively recognized Tn-expressing cells.

Sucheck and co-workers have also pursued three-component carrier protein-free TACA delivery strategies. In a 2010 disclosure,[46] the group described a synthetic vaccine construct incorporating the Tn B-cell epitope, a peptidyl T_h epitope (YAF peptide), and an immunoenhancing domain consisting of the rhamnose (Rha)

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carbohydrate epitope. This element was included on the basis of reports that human sera contain high titers of antirhamnose antibodies. Sucheck et al. hypothesized that incorporation of the Rha epitope on the vaccine backbone could serve to promote uptake by APCs, facilitating internalization of the vaccine construct, and accordingly, enhanced MHCII presentation. In a preliminary preclinical study, treatment of mice expressing anti-Rha antibodies with this vaccine construct did in fact lead to the production of anti-Tn antibodies. Control studies confirmed the critical role of the Rha domain in mediating the immune response.

Finally, Dumy et al. have explored the development of a four-component self-adjuvanting multivalent anticancer vaccine candidate.[47] The Dumy scaffold incorporates a B-cell epitope composed of a cluster of four Tn antigens, presented on a cyclic peptide scaffold. This domain is covalently linked to the peptidyl T_h (Pan-DR) and CTL (OVA) epitopes. Finally, a "built in" immunoadjuvant, palmitic acid (PAM) completes the chimeric vaccine scaffold. In mouse models, the Dumy construct was found to elicit IgG antibodies and, furthermore, to offer strong protection against tumor growth.

AUGMENTING THE CARRIER PROTEIN RESPONSE

Incorporation of Additional Immunoenhancing Elements. Our laboratory's efforts toward enhancing the T-cell response have primarily been directed toward augmenting, rather than replacing, the effect of the carrier protein. We describe below two bidomainal KLH conjugate platforms, designed with a view toward more effectively inducing T-cell-dependent immunogenic pathways.

Construct **18**, depicted in Figure 10, aims to evoke a strong T-cell response through installation of an MHC-II binding sequence in close proximity to the fucosyl-GM1 antigen. We reasoned that inclusion of this unit adjacent to the TACA domain might serve to increase the number of epitopes presented to the T_h cell CD4+ receptors. The synthesis of fuc-GM1-MHCII-BP-KLH has been accomplished,[48] and the results of immunological assays will be forthcoming.

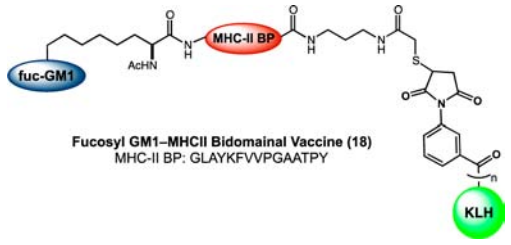

Figure 10. A fucosyl GM1-MHCII bidomainal vaccine.

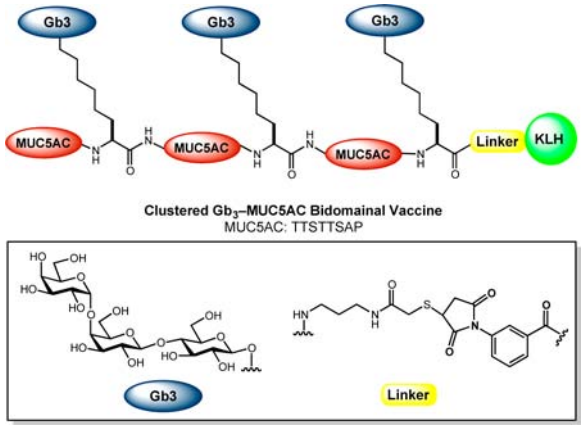

Figure 11. A clustered Gb_3–MUC5AC bidomainal vaccine.

In a separate effort, we hoped to exploit the immunogenicity of the mucin peptides. We reasoned that inclusion of a tumor-associated mucin sequence on the peptide backbone could enable an enhanced IgG antibody response. Along these lines, a clustered Gb_3-MUC5AC hybrid vaccine construct has been synthesized and conjugated to KLH (Figure 11).[49] Preliminary preclinical studies with this construct suggest a moderate IgM and IgG response to both the Gb_3 and MUC5AC antigens.

CONCLUSION

As described above, this program has been in motion for more than 20 years. Indeed, important advances have been registered, and one of our earlier constructs may well be approaching evaluation as to its registerability. We still look upon the effort as a work in progress. We feel that the ultimate challenge will surely center around the objective of creating a vaccine that will mobilize and synergize the full magic of the human immune system to do battle against cancer invasion.

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Notes

The authors declare no competing financial interest.

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