Developments in CRM$_{197}$ Glycoconjugates for Anticancer Vaccines

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Abstract

Glycoconjugate vaccines that utilize cross reactive material 197 (CRM$_{197}$) as an immunogenic carrier have shown clinical success against bacterial pathogens such as Haemophilus influenzae type b (Hib), Streptococcus pneumoniae, and Neisseria meningitidis. However, the translation of glycoconjugate vaccine strategies towards cancer has had limited clinical success. Preclinical efforts focused on tumor associated carbohydrate antigen (TACA)-CRM$_{197}$ conjugates include the Globo H, RM2, Thomsen-nouveau (Tn), Thomsen-Friedenreich (TF), and sialylated Thomsen-nouveau (STn) antigens. These collective efforts have shown robust conjugation chemistry and efficacious immune responses. The immune response towards these CRM$_{197}$ conjugates produced Gaussian-like distributions as a function of dose, with a maximum response at dosages in the low to medium range. This phenomenon suggests a tolerogenic effect toward the CRM$_{197}$ immunogen at high doses. An approach to resolve immune tolerance in cancer based glycoconjugate vaccines may be concomitant administration of TACA conjugates. As described by clinical research, the effects of simultaneous administration of antibacterial glycoconjugate vaccines have profound effects on immunological outcome.

Keywords: Cancer, Tumor Associated Carbohydrate Antigens, Glycoconjugates, CRM$_{197}$, Co-administered effects


Introduction

The concept of using glycoconjugates to induce immunological responses to a carbohydrate hapten was reported in 1929 by Avery and Goebel [1]. However, glycoconjugate vaccines would not be revisited as therapeutic and prophylactic agents against bacterial infections until the 1970’s and early 1980’s [2-4]. Prior to these interests in glycoconjugates, licensed antibacterial vaccines consisted of isolated capsular polysaccharides which were efficacious in adults but failed to induce protection in high risk populations such as infants and children [5,6]. Glycoconjugate vaccines consist of a bacterial capsular polysaccharide conjugated to an immunogenic carrier molecule such as keyhole limpet hemocyanin (KLH), diphtheria toxin (DT), tetanus toxoid (TT), cross-reactive material 197 (CRM197), as well as others [7-9]. This conjugate vaccine strategy is necessary due to the T cell independent nature of carbohydrates that have nominally elicited low affinity, short lived IgM antibodies with the exception of zwitterionic polysaccharides (ZPSs) [10-13]. Glycoconjugates are able to effectively bind to the MHCI and/or MHCII molecule on antigen presenting cells (APCs). This cellular interaction with the carbohydrate conjugate allows for the development of T cell mediated responses, and consequently leads to helper T cells (Th) which induce antibody isotype switching and immunological memory [14].

One of the more recent clinically successful carrier proteins in antibacterial glycoconjugates is CRM$_{197}$ [15]. CRM$_{197}$ is a nontoxic mutant of DT that has a single point mutation at position 52, which substitutes a glutamic acid residue with glycine [16]. The toxicity of DT is generated within a eukaryotic cell in which DT has ADP-ribosyltransferase activity towards elongation factor 2 (EF-2) causing a halt to protein synthesis, and consequently initiates cellular apoptosis. The single point mutation in CRM$_{197}$ limits this cytotoxic mechanism. Aside from being an inherently nontoxic
protein, CRM$_{197}$ also lacks lysine residues within its T cell epitope, meaning chemical conjugation of haptens will not likely effect T cell interactions [17]. There are currently CRM$_{197}$ glycoconjugate vaccines available for *Haemophilus influenzae* type b (Hib) (HBOC(HibTITTER), Pfizer, Inc.), *Streptococcus pneumoniae* (Prevnar 13, Pfizer, Inc.), and *Neisseria meningitidis* (Meneveo, Novartis Vaccines and Diagnostics, Inc.) [6,15].

The success of these bacterial glycoconjugate vaccines has helped develop similar strategies against certain epithelial cancers. Complementary to bacterial pathogens, cancer cells express a unique carbohydrate fingerprint as a result of genetic mutations that effect glycosyltransferase enzymes and chaperone proteins [18]. These unique tumor-associated carbohydrate antigens (TACAs) have been investigated within many glycoconjugate vaccines in conjunction with other approaches to involve immunological stimulation [7-9]. Unfortunately, these investigations have had limited clinical success as compared to the bacterial glycoconjugate vaccines.

**TACA-CRM$_{197}$ Glycoconjugates**

One of the first preclinical works on TACA-CRM$_{197}$ conjugates was published by Perico et al. [25] which described the synthesis of the terminal tetrasaccharide (Fucα(1→2)Galβ(1→3)GalNAcβ(1→3)Galβ) of the Globo H hexasaccharide [19]. Their linker strategy began with an allyl group at the reducing end which was oxidized to a methyl ester in 3 steps. The resulting methyl ester was then reacted with ethylene diamine which was designed to react with a bifunctional bis-hydroxysuccinimidy ester of adipic acid. This rather long linker was then used to conjugate to CRM$_{197}$'s many lysine residues resulting in an 11:1 ratio of hapten to protein. This conjugate, in comparison to CaMBr1-KLH conjugate, exhibited lower titers but was credited to give a more specific response towards the antigen as demonstrated by serum absorption experiments on non-CaMBr1 expressing cell lines [20]. The CRM$_{197}$ conjugate also displayed a dose response curve, highlighting a tolerogenic effect of the immunogen at higher doses. The results of these experiments were promising as they mirrored what has been observed in the clinical studies for bacterial glycoconjugate vaccines containing CRM$_{197}$.

Wong and coworkers would be the next to publish a series of works that involved TACA-CRM$_{197}$ conjugates [21]. The first one being that of the complete Globo H hexasaccharide (Fucα(1→2)Galβ(1→3)GalNAcβ(1→3)Galα(1→4)Galβ(1→4)Glcβ) moiety. The Globo H hexasaccharide featured a primary amine on the reducing end of the glycan with an allyl spacer. The free amine was then reacted with a p-nitrophenyl ester homobifunctional linker, which was further reacted with lysine residues on the CRM$_{197}$ protein to form a series of Globo H-CRM$_{197}$ conjugates with epitope ratios of 1.5, 5.1, 9.8, and 15.6. Interestingly, titers induced in mice gave a Gaussian-like response curve with Globo H5.1-CRM$_{197}$ giving the strongest IgG response. Furthermore, this conjugate, in comparison to the Globo H-KLH conjugate, gave equal or higher titer values, and the induced antibodies were more specific towards the target antigen as demonstrated by glycan array [21].

Shortly after Globo H, Wong et al. would publish another hexasaccharide, the RM2 antigen (GalNAcβ(1→4)(NeuAcα(2→3))Galβ(1→3)(NeuAcα(2→6))GlcNAcβ(1→3)Galβ) which has been discovered on prostate cancer cells, and its expression is closely associated with the prostate cancer staging Gleason grading system [22]. Similar to the Globo H approach, a 5-carbon spacer with a terminal amine was placed on the reducing end of the hapten. The terminal amine was first reacted with an N-hydroxysulfosuccinimide (sulfo-NHS) containing ester featuring an internal disulfide bond which was reduced after the formation of the RM2 antigen containing amide. Concurrently, the CRM$_{197}$ was reacted with the N-((maleimidocaproyloxy) sulfosuccinimide ester (Sulfo-EMCS) heterobifunctional linker which resulted in a thiol reactive maleimide functional group on the protein. Conjugation of the hapten to CRM$_{197}$ resulted in epitope ratios of 1.0, 3.0, 4.7, and 10. Furthermore, the immune response observed for these constructs displayed a Gaussian curve with a ratio of 4.7 being the most effective.

Highly desirable specificity was also demonstrated on a glycan array. In comparison, a series of mucin related CRM$_{197}$ conjugates were developed by Ye and coworkers featuring the Thomsen-nouveau (Tn) antigen (GalNAcα), the Thomsen-Friedenreich (TF) antigen (Galβ(1→3)GalNAcα), and the sialylated Thomsen-nouveau (STn) antigen (Neu5Acα(2→6)GalNAcα) [23-25]. The studies that featured the Tn and TF antigens focused on the modifications of the 2-N-acetyl moiety in each of the hapten structures which either displayed the natural N-acetyl structure, the propionyl, fluoroacetyl, difluoroacetyl, or trifluoroacetyl substituents [23,24].

These analogues contained an O-allylic group at the reducing end which was oxidized by ozone in methanol to produce an aldehyde intermediate. The aldehyde was then treated with the protein CRM$_{197}$ with aldehyde reactive lysine residues to form the conjugate through reductive amination. Conjugation of the Tn hapten led to glycoconjugates with hapten ratios ranging from 7.3 to 12.7 whereas the TF hapten led to hapten ratios between 4.0 to 11.2. Interestingly, the immune response was most prominent with the N-fluoroacetyl Tn-CRM$_{197}$ and N-fluoroacetyl TF-CRM$_{197}$ constructs within their respective groups. These studies were extended to the STn antigen with both N-acetyl groups on the STn structure modified to contain the N-fluoroacetyl functionality [25]. Similar trends were observed as the modification of the antigen enhanced both the antibody and cellular immune response to the natural antigen.

**Immune Tolerance Toward TACA-CRM$_{197}$ Glycoconjugates**

A direct comparison on the efficacy of these vaccine candidates can be rather difficult due to the number of variables present in each
individual study such as the different adjuvants chosen. In these studies, a number of adjuvants were used such as monophosphoryl lipid A (MPL-SE) [20], an analogue of α-galactosylceramide (C1) named C34 [21-25], and a combination of complete Freund’s adjuvant (CFA) and incomplete Freund’s adjuvant (IFA) [25]. The adjuvant C34 was used for the majority of the studies due to potent immune activating effects. As an analogue of C1, C34 targets the CD1d receptor on dendritic cells which has downstream effects to promote T cell activation, proliferation, IL-4 production, IFN-γ production, and an enhanced cytotoxicity [21]. These effects, stemmed from the CD1d-glycolipid-T cell receptor complex, promote a Th1 skew in T cell populations which are supposedly favored for anticancer responses. Another variable is the antigen loading within these glycoconjugates. Dose response curves for many successful vaccines created a Gaussian distribution with the most effective doses being within the low to medium range [26]. This was shown with Perico and coworkers as their CRM<sub>197</sub> conjugate gave good results at a dose of 2.5 μg rather than 0.5 μg or 12.5 μg suggesting a tolerogenic effect of the conjugate [19,20]. Similarly, Wong and coworkers demonstrated a Gaussian distribution of immunological responses when comparing the hapten to protein ratio [21,22]. Best results were observed with ratios of 5.1 and 4.7 rather than lower or higher ratios. These results suggest that there is a “Goldilocks” dosage for these glycoconjugates which also implies an immune tolerance at a high enough dose. The phenomenon involved with these observed dose response curves has been described as carrier induced epitope suppression (CIES). CIES involves a pre-existing immunity to the carrier which holds potential to suppress the immune response. These mechanisms may involve pre-existing antibodies that can bind to the conjugate causing steric hinderance, promotion of anti-carrier specific B cells over anti-hapten B cells, competition for resources when a anti-carrier B cells over populate anti-hapten B cells thus reducing hapten specific B cells from T cell help, and the production of regulatory T cells by the carrier [27,28]. This phenomenon is clearly observed in trials pertaining to anti-bacterial glycoconjugate vaccines where vaccines are concomitantly administered or serially administered [29,30].

Due to the clinical success of these anti-bacterial glycoconjugate vaccines and because these vaccines are usually administered concomitantly, investigations on vaccine interactions and co-administrative effects have revealed both enhanced and limited efficacy of these vaccines [28]. One such study by Borrow and coworkers involved the co-administration and the sequential administration of TT and CRM<sub>197</sub> conjugates which showed an enhanced immune response when conjugates were co-administered rather than administered sequentially, suggesting enhanced efficacy of a vaccine in the presence of another carrier [31]. In addition, another study performed by Dagan and coworkers involved the co-administration of a pneumococcal vaccine with an Hib vaccine using only TT as a carrier or both TT and CRM<sub>197</sub> [29]. When both conjugate vaccines utilized TT a bystander interference effect was observed resulting in decreased efficacy. However, this effect was not observed when both TT and CRM<sub>197</sub> was used.

**Conclusion**

Although CRM<sub>197</sub> has had clinical success in antibacterial glycoconjugate vaccines, CRM<sub>197</sub> glycoconjugates against cancer has had limited success. Most efforts to increase efficacy of TACA based glycoconjugate vaccines have been focused on creating non-natural analogues of the antigen, creating multivalent displays of a single antigen as well as multivalent displays of different antigens, discovering better adjuvants, and naturally occurring antibody recruiting epitopes [7-9]. However, there are limited studies that have observed co-administration effects of TACA-CRM<sub>197</sub> conjugates as compared to antibacterial CRM<sub>197</sub> glycoconjugates.

The co-administrative effects in bacterial glycoconjugate vaccines are still not well understood, but there is consensus that there are both positive and negative effects and that these effects differ in vulnerability [28]. Concomitant vaccine effects may also be translated into preclinical research involved with TACA-CRM<sub>197</sub> conjugates where the co-administration of TACA conjugates could have a profound effect on observed immunity. Following the few examples herein, using CRM<sub>197</sub> in combination with another immunogenic carrier may have beneficial effects and enhance immunity towards a single hapten structure and reduce tolerogenic effects observed in TACA-CRM<sub>197</sub> preclinical studies [19-22]. Such examples could include the combination of KLH, TT, or ZPSs [10-13,32,33].

**References**


