Synthetic carbohydrate ligands for immune receptors

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Chapter 7

Summary and future prospects

Summary

One of the main challenges in the development of an effective anti-cancer vaccine is the generation of an adequate and directed cellular immune response. Therefore, much attention has been directed to the construction of vaccine modalities that target dendritic cells (DCs), as these interact with both B cells and T cells, mediating humoral and cellular immune responses. DCs express pathogen recognition receptors (PRRs), that recognize pathogen associated molecular patterns, and Fc receptors that can bind antibodies complexed to antigens on the pathogens. These two types of receptors have been exploited in the development of novel vaccine modalities.[1,2] Ligands for PRRs, such as Toll-like receptors (TLRs) and Nucleotide binding oligomerization domain (NOD)-like receptors, can be used as vaccine adjuvants as they can induce maturation of DCs, stimulate the production of co-stimulatory molecules, and upregulate antigen presentation via MHC molecules. Combinations of specific antigens and selected PRR-ligands are widely investigated in vaccine modalities to generate a directed and improved immune response. Special examples are represented by the development of

synthetic peptide conjugate vaccines, in which a peptide antigen is covalently connected to one or more structurally defined PRR ligands. In the second strategy, Fc receptors are exploited as they bind to an immune complex, which is formed by binding of an antibody to an antibody-recruiting molecule (ARM). Antigens equipped with ARMs can thus be taken up more efficiently by DCs leading to enhanced antigen presentation and a more adequate immune response. The conjugation of a PRR-ligand or an ARM to an antigen is thus a much explored strategy to enhance the immunogenicity of vaccines and selected examples of immunostimulants and vaccine conjugates have been described in **Chapter 1**.

Chapter 2 describes the design, synthesis and immunological evaluation of four TLR4-ligand peptide-conjugates. In these conjugates CRX-527, a monophosphoryl lipid A analogue, was used as the built-in adjuvant, as it represents a powerful TLR4 stimulating agent, of which the mechanism of action is well described. In the generation of the conjugates several synthetic challenges had to be overcome. First, the route of synthesis towards CRX-527 was optimized and a route was developed that allowed the multi-gram scale synthesis of the required (R)-3-alkyloxytetradecanoic acids. The introduction of the chiral fatty acid on the glucosaminyl serine building block is a key step, and a silylidene protected glucosamine building block is introduced in Chapter 2 that allows for the successful incorporation of multiple fatty acids in the target structure and enables an effective purification of the generated lipid carrying carbohydrate. To investigate the influence of the linker on the immunological properties of CRX-527, three different ligands were generated, equipped with a hydrophobic or a hydrophilic linker, connected to the *C*-6 position of the glucosamine core via an ester or amide bond. The ligand equipped with a hydrophobic linker was incapable of inducing the production of the pro-inflammatory interleukin-12 (IL-12), likely because the addition of the extra fatty tail to the structure prevents proper binding to the MD2-TLR4 complex. Therefore, the ligand equipped with a hydrophilic spacer was used to generate conjugates with the ovalbumin derived $DEVA_5K$ peptide, containing the MHC-I epitope SIINFEKL. Using thiol/maleimide chemistry, four conjugates were assembled in which the ligand was either connected to the *N*- or the *C*-terminus of the peptide. A manual reversed phase chromatography protocol had to be developed, as the ester bonds at the *C*-6 position of CRX-527 turned out to be acid and base labile, prohibiting HPLC purification. Stimulation of DCs with the four new conjugates resulted in a higher IL-12 production for the ester conjugates, while the amide conjugates showed enhanced antigen presentation *in vitro*. The four new conjugates prove to be promising

self-adjuvanting vaccine modalities and further *in vivo* evaluation of the conjugates is currently ongoing.

Chapter 3 describes the exploitation of muramyl dipeptide (MDP), a NOD2 ligand, in four MDP-HPV-conjugates and four MDP/TLR2-ligand bisconjugates. With the aid of solid phase peptide synthesis (SPPS), a suitably protected *O*-MDP building block, containing an *N*-acetyl or an *N*-glycolyl group, was connected via its isoglutamic acid moiety to an HPV-16 derived peptide, containing both an MHC-I and an MHC-II epitope. An orthogonal protected lysine at the *C*-terminus of the immobilized peptide was used to introduce the TLR2-ligand, Pam_3CSK_4, leading to the projected bis-conjugates. In the second part of this Chapter, two *C*-glycoside MDP analogues bearing either an *N*-acetyl or *N*-glycolyl moiety, were synthesized. Key steps in the synthesis of these two *C*-MDP building blocks are the installation of the double bond on the glucosamine core, a Grubbs cross metathesis to install the carboxylic acid conjugation handle, and the subsequent reduction of the obtained alkene. The acid stability of the *C*-MDP analogues allowed their coupling to the immobilized peptide, via a spacer at the anomeric position, using a SPPS approach, leading to new NOD2-ligand HPV-conjugates and bisconjugates.

The mannose-6-phosphate receptor (MPR) is able to recognize and bind mannose-6-phosphate present on newly synthesized proteins and to deliver these to the endosomes. It also shuttles to the cell surface where it can take up mannose-6-phosphate carrying cargo. It was speculated that this pathway could be exploited to enhance the uptake of mannose-6-phosphate bearing peptide antigens, thereby leading to a stronger immune response. **Chapter 4** describes the synthesis of two mannose-6-phosphonate building blocks, an *O*-analogue (*O*-M6Po) equipped with an anomeric alkyne spacer and a *C*-mannosyl analogue (*C*-M6Po) equipped with an anomeric lysine spacer. In these building blocks the natural phosphate at the 6-position of the mannose moiety has been replaced with a *C*-phosphonate to prevent dephosphorylation by phosphatases. The installation of the phosphonate turned out to be critically depended on the protecting groups present on the mannosyl synthon, and it was revealed that an 2,3-*O*-isopropylidene group was required to prevent an intramolecular cyclization side reaction. Six *O*-M6Po and *C*-M6Po building blocks were incorporated at the *N*-terminus or *C*-terminus of the antigenic peptides, containing either a CTL epitope or a Th epitope, resulting in four multivalent M6Po-conjugates. The six *O*-M6Po residues were appended in one event to the separately prepared peptides by a copper mediated 1,3-dipolar cycloaddition reaction. The *C*-M6P building block

proved to be well suited for SPPS allowing an online SPPS of the projected conjugates. With the objective to further enhance the immunogenicity, four bisconjugates were designed and synthesized, in which not only the M6Po-ligands were incorporated but also a TLR7-ligand to ensure the activation of endosomal expressed TLR7.

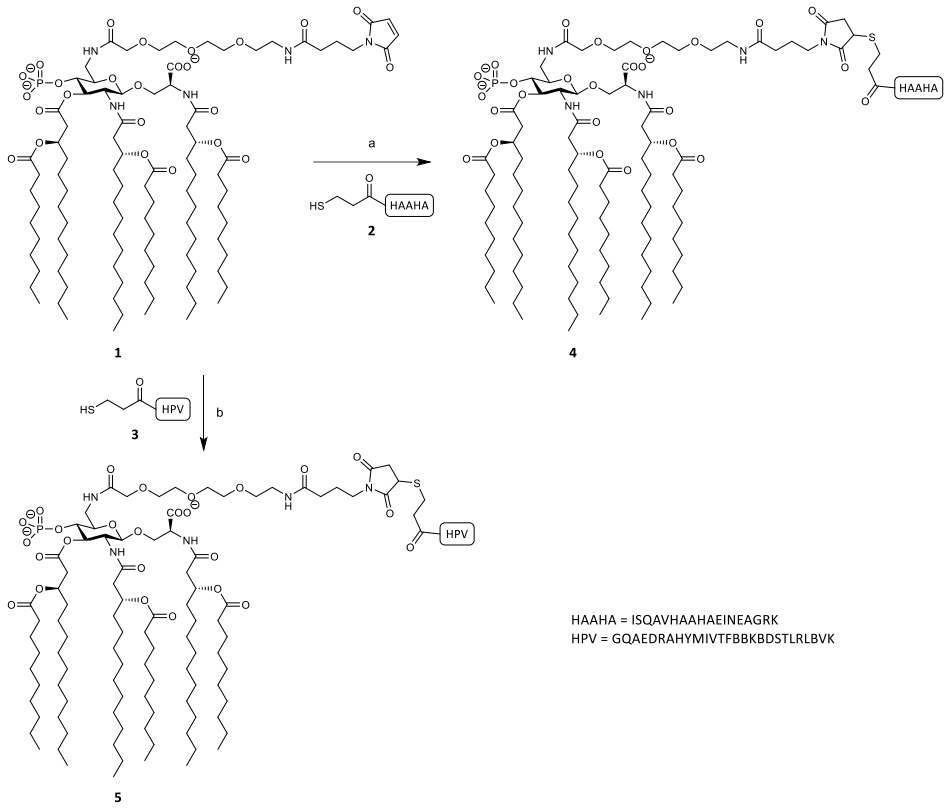
Chapter 5 describes the synthesis of seven novel rhamnose-peptide conjugates via an online SPPS. The design of these multivalent conjugates is based on rhamnose as an antibody recruiting molecule to generate antibody complexes that can be taken up by DCs through binding to Fc receptors. The generated conjugates carried one, two, three or six rhamnose moieties connected to an ovalbumin derived peptide LEQLESIINFEKLAAAAAK, harboring the MHC-I epitope SIINFEKL. An acid stabile *C*-analogue of rhamnose was used in the synthesis of *C*-rhamnose building blocks in which the rhamnose monosaccharide was connected to a lysine residue through a *C*-butanoic acid or an extended PEG-spacer, leading to two new building blocks suitable for SPPS chemistry.

Chapter 6 describes the design and synthesis of four *C*-glycosyl lysine building blocks, which can be used in standard SPPS. These building blocks are functionalized with either an α-mannose, β-*N*-acetyl-glucosamine, β-galactose or α-galactose residue and the synthons were protected with acid-labile protecting groups. The protective group pattern allows the concomitant removal of all acid labile (glyco)peptide protecting groups as well as the release of the peptide from the resin, at the final stage of the SPPS. Key steps in the synthesis of these building blocks are the Grubbs cross-metathesis, reduction of the double bond and subsequent hydrolysis of the methyl ester to allow the connection at the side chain of lysine.

Future prospects

The TLR4-ligands described in **Chapter 2** can be further exploited for incorporation in new conjugates with other epitopes. The first endeavors that have been undertaken include conjugation to the ovalbumin derived (HAAHA) peptide, which contains an MHC-II epitope, and the HPV-16 derived peptide, harboring both MHC-I and MHC-II epitopes (Scheme 1). The latter can be used as a vaccine against the human papillomavirus (HPV), which is responsible for cervical cancer. Both conjugates were synthesized via thiol-maleimide coupling of ligand **1** to peptides **2** and **3** (Scheme 1). The purification method developed in Chapter 2, only proved effective for the purification of conjugate **4** and therefore conjugate **5** was purified by RP-HPLC.

Conjugates **4** and **5** were obtained in respectively 40% (1.4 mg) and 54% (2.2 mg). At present, the immunological evaluation of these two conjugates is ongoing.



Scheme 1. Synthesis of TLR4-ligand peptide conjugates **4** and **5**. *Reagents and conditions*: a) **2**, DMF/$CHCl_3$/H_2O, 72h, 40%; b) **3**, DMF/$CHCl_3$/H_2O, 72h, 54%.

Tada *et al*. have studied the synergy between different PRR ligands and they discovered that a mixture of TLR4-ligands and NOD-ligands can enhance the immune response with over a 1000-fold compared to stimulation with the separate ligands.[3] Therefore, it would be interesting to synthesize bisconjugate **6** (Figure 1) in which the TLR4-ligand (**Chapter 2**), the NOD2 ligand MDP (**Chapter 3**) and an antigenic peptide are covalently connected. To further enhance the immunogenicity a third PRR-ligand may be incorporated, by coupling the TLR2-ligand Pam_3CSK_4 after deprotection of an MMT-protected lysine at the *C*-terminus, resulting in triple-conjugate **7**. This conjugate contains all the main components of the bacterial cell wall that are recognized by PRRs: TLR4, TLR2 and NOD2. The orthogonally protected *C*-MDP building blocks, described in **Chapter 3** can also be used to introduce multiple MDPs in an antigenic peptide, as in

conjugates **8** and **9** (Figure 1). The multivalent MDP-conjugates could lead to better binding to NOD2, thereby enhancing the potency of the conjugate. In order to bind to the NOD2 receptor, the MDP has to enter the cytosol and to improve this transport a cell-penetrating peptide (CPP), such as GRKKRRQRRRPSQ, could be incorporated in the conjugates as shown in **10** and **11**.[4,5]

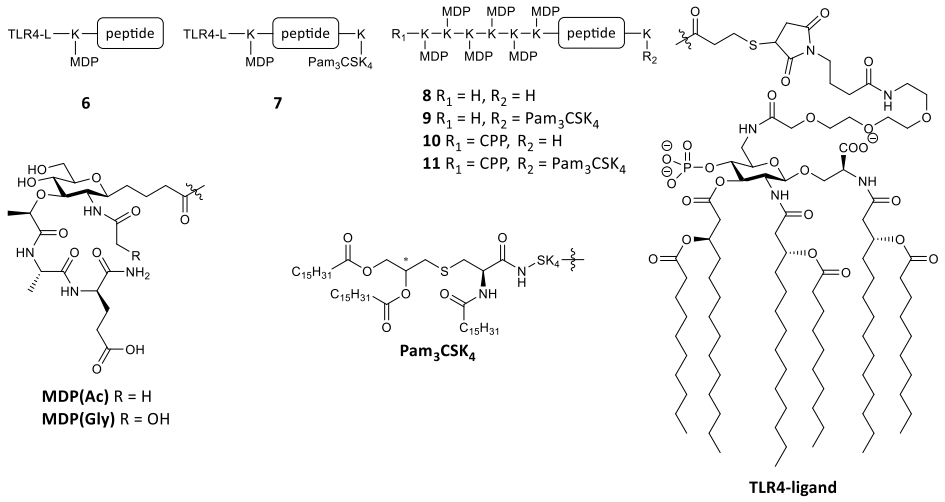


Figure 1. Proposed conjugates **6**-**11**.

Gold nanoparticles (AuNPs) have been shown to be effective carriers to improve vaccine efficacy.[6,7] AuNPs offer the possibility to combine various components needed to develop a vaccine in a controlled way and thereby deliver all immunological signals in a single well-defined system.[8] The repetitive surface organisation of AuNP facilitates phagocytosis, resulting in better processing of the antigens and subsequent activation of the immune system.[9,10] The potential of AuNPs as candidates for vaccine carriers is exemplified by their use in vaccine-modalities against *Streptococcus pneumonia*, HIV or cancer.[10–13] Therefore, it is interesting to use the NOD-2 ligands, described in **Chapter 3**, for the development of MDP-coated AuNPs. The multivalency of AuNPs[14] leads to clusters of several MDP molecules on the surface that may resemble MDP "clusters" of the bacterial cell wall. To address the influence of particle size[15] and ligand density on the immunoactivity of MDP-coated AuNPs, four 5 nm particles (**12**-**15**, Figure 1) were prepared with either a low or a high concentration of MDP(Ac) and MDP(Gly), and for the generation of 2 nm particles (**16**-**19**, Figure 2), two different ratios (1/1 and 1/9) between MDP and glucose, as an inert "inner component", were used.