Multivalent Vaccines Hot Paper

DOI: 10.1002/anie.201308875

Synthetic Multivalent Glycopeptide-Lipopeptide Antitumor Vaccines: Impact of the Cluster Effect on the Killing of Tumor Cells**

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Abstract: *Multivalent synthetic vaccines were obtained by solid-phase synthesis of tumor-associated MUC1 glycopeptide antigens and their coupling to a Pam_3Cys lipopeptide through click reactions. These vaccines elicited immune responses in mice without the use of any external adjuvant. The vaccine containing four copies of a MUC1 sialyl-T_N antigen showed a significant cluster effect. It induced in mice prevailing IgG_{2a} antibodies, which bind to MCF-7 breast tumor cells and initiate the killing of these tumor cells by activation of the complement-dependent cytotoxicity complex.*

Choosing sufficiently tumor-specific antigens is a prerequisite for the development of an efficient antitumor vaccine.[1] Tumor-associated membrane glycoproteins are considered most important antigens for vaccine development.[2] One of the major problems opposing the development of an efficient antitumor vaccine is the insufficient immunogenicity of these endogenous antigens. Chemical synthesis is a powerful method not only for the preparation of complex glycopeptide antigens with specific glycosylation modification,[3] but also for combining glycopeptide antigens with immune stimulants, which are essential to enhance and direct the immune response against the antigens.[4] Generally, antigens such as glycopeptides are covalently conjugated with carrier proteins to enhance the immune reactions.[5] Alternatively, the bacterial lipopeptide Pam_3-Cys-Ser-Lys_4 (Pam_3CSK_4), a ligand of Toll-like receptor 2 (TLR2),[6] was used for conjugation with glycopeptides in order to amplify the immune response.[7] Recently, a glycopeptide antigen, a T-cell epitope, and the lipopeptide Pam_3CSK_4 were covalently combined to give fully synthetic three-component vaccines.[8] In our previous studies, efficient antitumor vaccines were obtained by the conjugation of MUC1 glycopeptides with the carrier proteins bovine serum albumin (BSA),[9] and, in particular, tetanus toxoid.[10] In order to prevent immune responses towards antigens of the carrier protein, we also conjugated MUC1 glycopeptides with a T-cell epitope peptide[11] and with the lipopeptide Pam_3CSK_4, and thus obtained fully synthetic two- and three-component vaccines.[12] Meanwhile, multivalent MUC1 glycopeptides vaccines were obtained by self-assembling structures or by presentation on polymers.[13]

To combine the glycopeptide antigen and the immune adjuvant within a unimolecular structure, we now synthesized and evaluated multivalent antitumor glycopeptide vaccines that contain the TLR2 lipopeptide Pam_3CSK_4 combined with up to four MUC1 tandem repeat glycopeptide antigens carrying Tn or sialyl-Tn (STn) tumor-associated carbohydrate antigens (TACAs). Based on the multiple antigen peptide (MAP) strategy,[14] the syntheses were achieved by coupling the MUC1 glycopeptides to a multibranched lysine core through Cu^I-mediated click reactions.[15] The multibranched lysine core was linked to the intact TLR2 ligand lipopeptide Pam_3CSK_4 (Figure 1).[16]

The lipopeptide $Pam_3CysSer$-$(Lys)_4$-OH was synthesized as previously described.[7c,16] To keep the Pam_3CSK_4 structure[17] intact during the conjugation to the glycopeptides, an additional lysine was inserted at the C-terminus of the lipopeptide. It served for the coupling with the branched oligolysine core at its ε-amino group. The alkyne-functionalized oligoethylene glycol spacers were linked to the lysine core. In parallel, the glycopeptide antigen **1** with two Tn antigens linked at T9 and S15, and **2** with STn at S15 of MUC1 tandem repeat domain HGVTSAPDTRPAPGSTAPPA were synthesized on solid phase as described previously[7c,9a,10a] and N-terminally acylated with an azido-functionalized spacer carboxylic acid.[16,18] These glycopeptides were released from the resin using trifluoroacetic acid/triisopropylsilane/water. Subsequently, the protecting groups were removed from the carbohydrate portions (Scheme 1). It should be emphasized

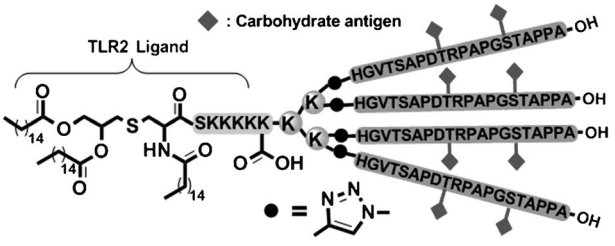

Figure 1. Structure of a vaccine containing the TLR2 ligand lipopeptide conjugated to tetravalent MUC1 glycopeptides.

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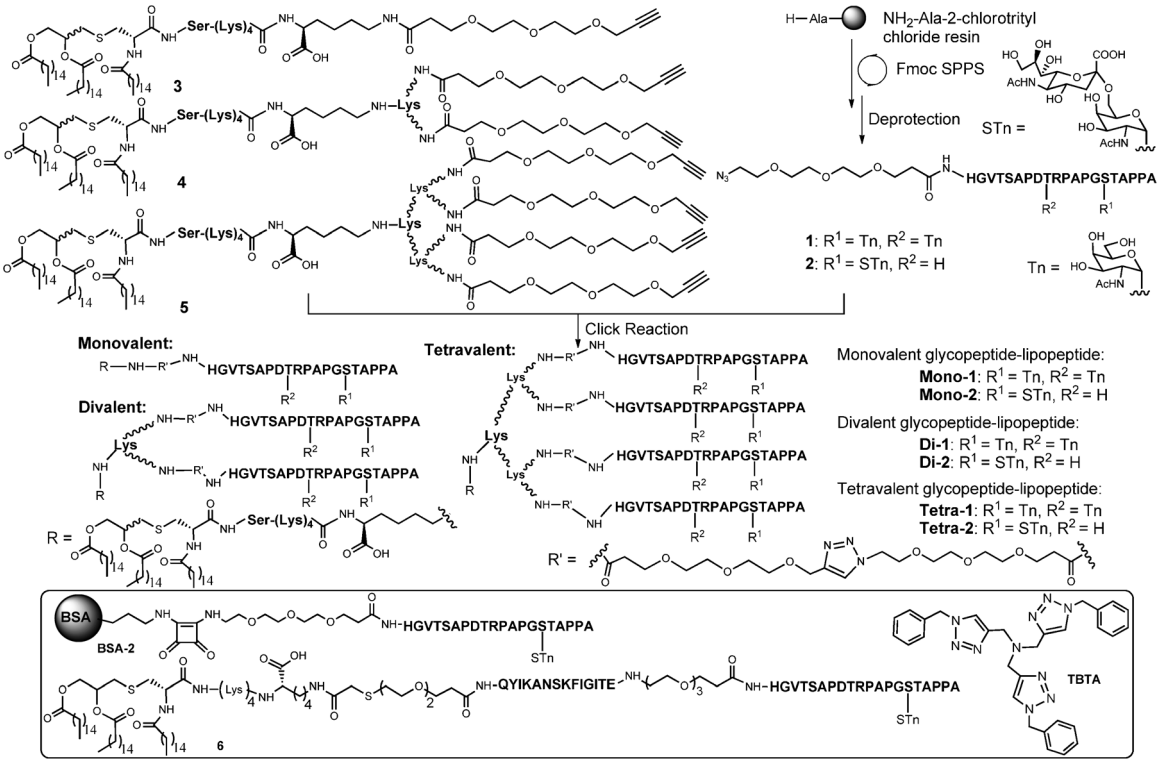
[**] This work was supported by the Major State Basic Research Development Program of China (2013CB910700 and 2012CB821601), the National Natural Science Foundation of China (21332006, 91313301, and 21028004), and the Sino-German Center for Research (GZ561).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201308875.



Scheme 1. Structure of the fully synthetic mono-, di-, and tetravalent glycopeptide-lipopeptide vaccine candidates.

that the removal of the sialic benzyl ester from glycopeptide **2** was achieved concomitantly with O-deacetylation by treatment with aq. NaOH at pH 11.4.[10a] After purification by HPLC, these glycopeptides were conjugated to the mono-, di-, and tetraalkyne-functionalized lipopeptides (**3**, **4**, and **5**) through click chemistry[15] to afford the mono-, di-, and tetravalent glycopeptide-lipopeptide vaccine candidates Mono-**1**, Mono-**2**, Di-**1**, Di-**2**, Tetra-**1**,[16] and Tetra-**2**. The immunological investigation of vaccines containing glycopeptide **2** (Mono-**2**, Di-**2**, and Tetra-**2**) appeared interesting as the corresponding BSA conjugate BSA-**2** had induced antibodies with low affinity towards tumor cells in a previous study.[9a] In addition, a three-component vaccine **6** including the Pam_3CSK_4, the T-cell epitope peptide P2 QYKANSKFIGITE derived from tetanus toxoid, and the MUC1 glycopeptide **2** glycosylated with STn was synthesized through thioether ligation[12b] in order to compare its immunological effects with those induced by vaccines Mono-**2**, Di-**2**, and Tetra-**2**.

To evaluate the immune responses elicited by these synthetic vaccines, four Balb/c mice were immunized with 10 μg of each vaccine Mono-**2**, Di-**2**, Tetra-**2**, and **6** in phosphate buffer saline (PBS) by intraperitoneal injection without the aid of an external adjuvant. Booster immunizations were performed at weeks 2, 4, 6 and 8. One week after the fifth immunization, blood was drawn from the tail vein of the mice, and the sera from each group of mice were mixed and investigated by enzyme-linked immunosorbent assay (ELISA) for induced total antibodies. The antisera induced by vaccine Di-**2** showed the highest titers towards the MUC1 glycopeptide conjugated to BSA (Scheme 1), while lower titers were recorded for the antisera induced by Tetra-**2** and the three-component vaccine **6** (see the Supporting Information). However, all these titers were lower than those which had been induced by the corresponding BSA-conjugated vaccine.[9a] In order to exclude that the immune response induced by the tumor-associated antigens may be overwritten by that induced through triazole linker structures,[19] the extent of the immune response against the linker was evaluated by ELISA on plates coated with tris[(1-benzyl-1*H*-1,2,3-triazole-4-yl)methyl]amine (TBTA) (Scheme 1), which contains three triazole residues and is sufficiently insoluble in water[20] to form the coat. No binding of the antisera to the triazole residue was observed (see the Supporting Information).

The evaluation of the antisera binding to antigens exposed on cells of human breast tumor cell line MCF-7 was carried out by flow cytometry. The MCF-7 cells were incubated with the antisera diluted to 1:50. The mouse antibodies bound to the cells were stained by FITC-conjugated rabbit anti-mouse IgG antibodies. This experiment surprisingly showed that the antisera induced by vaccine Tetra-**2** exhibited the strongest binding to MCF-7 cells (Figure 2), while the antisera induced by Mono-**2**, the three-component vaccine **6** (see the Supporting Information), and the BSA-conjugated vaccine[9a] all show weak binding.

Antibodies of the induced antisera that can bind to the MCF-7 tumor cells are supposed to initiate the killing of the marked tumor cells through activation of the complement-dependent cytotoxicity (CDC) complex. There are three pathways of CDC activation: the classical, the lectin-like, and the alternative pathways.[21] As the lectin-like pathway

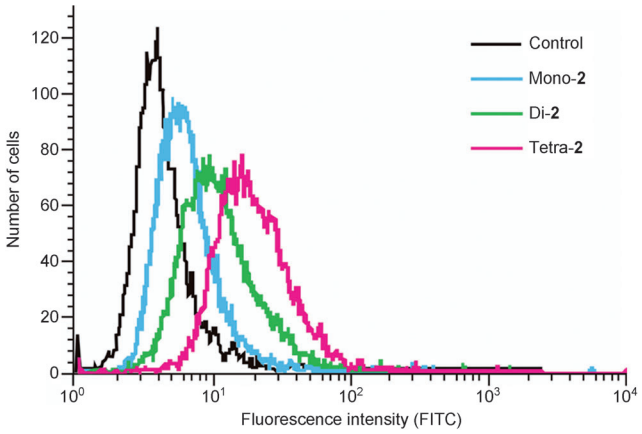


Figure 2. FACS analysis of the binding of antisera induced by Mono-/Di-/Tetra-**2** to MCF-7 tumor cells.

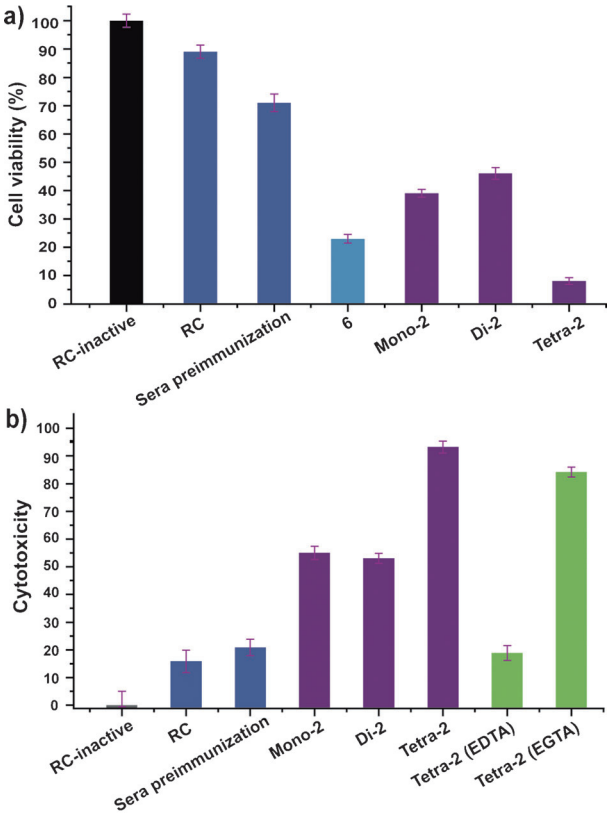


Figure 3. Cell viability and cytotoxicity assay. a) MTT assay measuring the complement-dependent cell viability. b) LDH assay measuring complement-dependent cytotoxicity. RC: rabbit complement. RC-inactive: rabbit complement, inactivated by treatment at 65 °C for 30 min. EDTA and EGTA were used as inhibitors of the complement-dependent cytotoxicity. Data shown are the mean of five independent experiments. Student's t-test, $P < 0.01$.

involves carbohydrate-recognizing receptors, either mannose-binding lectin or ficolins,[22] the activation pathway initiated by the antisera induced from MUC1 glycopeptide vaccines probably proceeds by the classical or the alternative pathway. Both of these pathways finally form the membrane-attacking complex (MAC) which plunges pores in the membrane and leads to lysis of the cells.[23] To answer these questions, MCF-7 tumor cells were incubated with the antisera and planted on the plates. After the cells had been washed with PBS, rabbit sera were added as complement supplier (RC: rabbit complement). The survival rate of the tumor cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay, and the mortality rate was recorded by the lactate dehydrogenase (LDH) assay in parallel. As shown in Figure 3, the antisera induced with vaccine Tetra-**2** led to more than 90% cell death. It mediated distinctly stronger activation of CDC than the antisera induced by the three-component vaccine **6**. Compared with the analogous vaccines Mono-**2** and Di-**2**, the antiserum induced from Tetra-**2** also resulted in stronger cytotoxicity against MCF-7 tumor cells. These results were confirmed by the LDH assay (Figure 3b).

The CDC activation pathways were differentiated by inhibition tests using ethylene diamine tetraacetic acid (EDTA) and ethylene glycol bis(2-aminoethylether) *N,N,N′,N′*-tetraacetic acid (EGTA). EDTA traps both Mg^{2+} and Ca^{2+} cations, and consequently inhibits both the classical and the alternative pathways,[24] while EGTA can selectively trap Ca^{2+} cations and blocks only the classical pathway. As shown in Figure 3b, addition of 10 mM EDTA to the culture medium almost completely inhibited the CDC; however, addition of 10 mM of EGTA did not inhibit the CDC. This indicates that the alternative pathway of the CDC activation is predominant.

The antisera induced by vaccine Tetra-**2** displayed the strongest tumor cell recognition (Figure 2) and initiated the most extensive killing of tumor cells through CDC (Figure 3). These results were surprising, since the titer of the antisera induced by Tetra-**2** had been relatively moderate. To find out the reasons for these unexpected results, ELISA tests were performed to analyze the isotypes of the antibodies induced by the vaccines. As shown in Figure 4, IgG2a antibodies were predominant in the antisera induced by Tetra-**2**. The IgG2a/IgG1 ratio was greater than 2.0 at a dilution of 1:800. In the antisera induced by Mono-**2** and Di-**2**, the amounts of IgG1 and IgG2a were almost equal. The tetravalent glycopeptide-lipopeptide vaccine predominantly elicited IgG2a antibodies which obviously are responsible for the more potent killing effect. This may be traced back to an increased cluster effect. To prove this assumption, the antibody isotypes of antisera induced by vaccine Mono-/Di-/Tetra-**1** were also evaluated and similar results were obtained. IgG2a antibodies were predominant in the antisera induced by the clustered vaccines Di-**1** and Tetra-**1** (see the Supporting Information and Figure 4e).

This interesting change in the antibody isotype pattern and its influence on the killing of the tumor cells has been discovered for the first time for the synthetic vaccines described herein, which expose different numbers of the identical STn glycopeptide antigens that are linked to the TLR2 ligand lipopeptide Pam_3CSK_4. Except for one example,[25] these are the first multivalent MUC1 glycopeptides with the STn antigen. They were immunologically evaluated without using external adjuvants during the immunization.

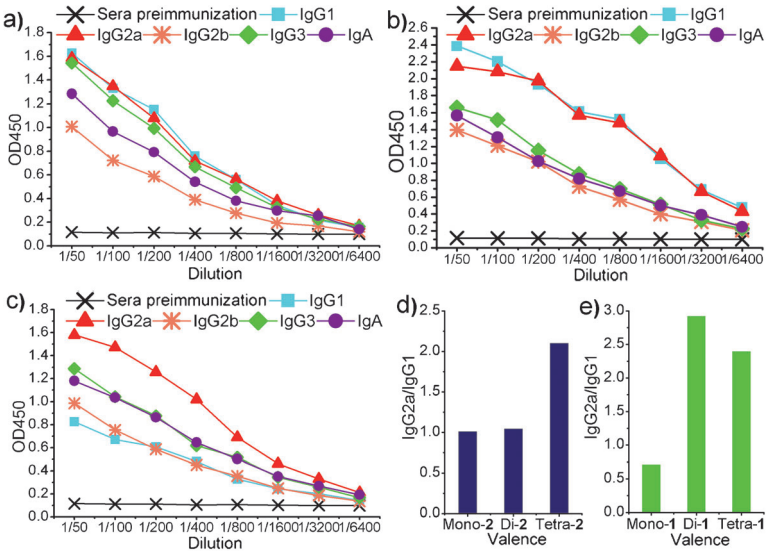


Figure 4. Antibody isotype analysis. a)–c) Antibody isotype analysis of the antisera induced by Mono-**2** (a), Di-**2** (b), and Tetra-**2** (c). d) IgG2a/IgG1 ratios elicited by Mono-, Di-, and Tetra-**2**, which were determined by the OD value of the titer at a dilution of 1:800. e) IgG2a/IgG1 ratios elicited by Mono-, Di-, and Tetra-**1**, which were determined by the OD value of the titer at a dilution of 1:800. For details of the antibody isotypes of the antisera induced by Mono-, Di- and Tetra-**1** see the Supporting Information.

According to the ELISA tests, the induced antibodies do not recognize the triazole structures of the linkers present in these synthetic vaccines, since the antibodies did not bind to TBTA containing three triazole residues. Therefore, the immunogenicity of the glycopeptide was not overwritten by that of the linker included in the vaccines. The immune responses induced by vaccines Mono-**2**, Di-**2**, and Tetra-**2** did not disclose a clear cluster effect as concluded from the ELISA analysis. The divalent vaccine Di-**2** elicited the strongest titer, while the tetravalent vaccine Tetra-**2** induced the lowest antibody titer against the antigen, which was even lower than the titer induced by the three-component vaccine **6**. However, the antisera induced by vaccine Tetra-**2** exhibited the strongest binding to the MCF-7 tumor cells (Figure 2). The binding to MCF-7 tumor cells increased with increasing glycopeptide valency. This result is in marked contrast to previous results observed with antibodies induced by the BSA-conjugated vaccine[9a] containing the identical MUC1 glycopeptide antigen. Moreover, the antibodies bound to the tumor cells caused very strong cell cytotoxicity by initiating the killing of the recognized tumor cells through activation of CDC, mainly by the alternative pathway (Figure 3). The antibody isotype analyses of the antisera disclosed that from Mono-**2** to Di-**2** and Tetra-**2**, the predominant antibody isotype changed from IgG1 to IgG2a. This is illustrated by the IgG2a/IgG1 ratio (Figure 4). Although low titers were observed for the immune response elicited by the tetravalent vaccine Tetra-**2**, its cluster effect resulted in a marked increase of the isotype ratio IgG2a/IgG1. A similar trend was observed for the Mono-**1**, Di-**1**, and Tetra-**1** vaccines. The generation of IgG1 antibodies is usually associated with Th2 responses, whereas high levels of IgG2a are thought to reflect Th1 responses.[26] Therefore, the IgG2a/IgG1 ratio is considered to reflect the relative contribution of the Th1/Th2 pathways to the immune response.[27] Thus, the cluster effect of these glycopeptide vaccines resulted in an increased contribution of Th1 response pathway during the immune reaction, and the high level of IgG2a antibodies is responsible for the enhanced binding to tumor cells and the cytotoxic effects.

In conclusion, the tetravalent MUC1 glycopeptide-lipopeptide vaccine Tetra-**2** not only induced a sufficiently strong antiserum binding to tumor cells, but also elicited an antiserum that initiated an efficient CDC-mediated killing of the tumor cells. Both the binding and cytotoxicity were enhanced with the increasing numbers of STn-bearing glycopeptide units. The sera induced by the Tetra-**2** exhibited stronger binding and cytotoxicity than those induced by the corresponding BSA conjugate and by the three-component vaccine. Furthermore, it was disclosed that the CDC was activated by the alternative pathway. These effects are considered particularly important for therapeutic applications.

Received: October 11, 2013
Published online: January 21, 2014

Keywords: antigens · antitumor agents · cytotoxicity · multivalent glycopeptides · vaccines

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