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Induction of a Melanoma-Specific Antibody Response by a Monovalent, but not a Divalent, Synthetic GM2 Neoglycopeptide

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The GM2 ganglioside represents an important target for specific anticancer immunotherapy. We designed and synthesized a neoglycopeptide immunogen displaying one or two copies of the GM2 tetrasaccharidic moiety. These glycopeptides were prepared using the Huisgen cycloaddition, which enables the efficient ligation of the alkyne-functionalized biosynthesized GM2 with an azido $CD4^+$ T cell epitope peptide. It is worth noting that the GM2 can be produced on a gram scale in bacteria, which can be advantageous for a scale-up of the process. We show here for the first time that a fully synthetic glycopeptide, which is based on a ganglioside carbohydrate moiety, can induce human tumor cell-specific antibodies after immunization in mice. Interestingly, the monovalent, but not the divalent, form of GM2 peptide construct induced antimelanoma antibodies. Unlike traditional vaccines, this vaccine is a pure chemically-defined entity, a key quality for consistent studies and safe clinical evaluation. Therefore, such carbohydrate–peptide conjugate represents a promising cancer vaccine strategy for active immunotherapy targeting gangliosides.

Introduction

GM2 is a ganglioside consisting of a sialylated tetrasaccharide [GalNAcβ-4(NeuAcα-3)Galβ-4Glc] linked to a ceramide tail. It is well established that this GM2 antigen is overexpressed in various types of cancer, including breast, ovary, prostate, small cell lung cancer and melanoma.[1] GM2 was first identified as a target for immunotherapy when tumor regression was observed in melanoma patients after passive immunotherapy with GM2-specific monoclonal antibodies (mAbs).[2] Furthermore, GM2-induced antibodies (Abs) have been shown to mediate complement-dependent cytotoxicity (CDC) for human GM2+ tumor cells.[3,4] Finally, the level of serum IgM Abs recognizing the GM2 ganglioside was correlated with an improved survival of stage III melanoma patients.[4,5]

These clinical observations have strongly motivated the development of anticancer vaccines targeting the GM2 on tumor cells.[6] Indeed, intensive efforts have been directed toward the preparation and evaluation of GM2-derived immunogens, most notably by Livingston and co-workers. The use of the KLH carrier protein together with the immunological adjuvant QS21 has proved optimal for increasing the antibody response against the GM2 carbohydrate antigen in both mice and humans.[7–10] In melanoma patients, the KLH protein conjugate consistently induced high titers of GM2-specific IgM, followed by a long lasting IgG response.[8,11] Furthermore, these anti-GM2 antibodies were shown to react with GM2+ tumor cells and to induce CDC.[11,12]

Based on these successful results, a randomized phase III clinical trial was initiated in patients with resected stage IIB–III melanoma. When coadministered with QS21, treatment with the GM2–KLH conjugate was found to be no more effective than interferon-α2b, the current standard therapy for high-risk melanoma.[13] Nevertheless, the two phase III clinical trials conducted so far with GM2 vaccines have suggested a clinical advantage in patients with enhanced GM2 antibodies.[4,13]

To our knowledge, all the GM2 vaccines subjected to clinical trials so far have been prepared with a ganglioside extracted from bovine or cat brains.[4,7,8,11,13] This animal origin is of major concern for human use when safety issues are considered. Additionally, it is well known that isolation of carbohydrate antigens from natural sources often leads to heterogeneous and low amounts of products.

Fortunately, great progress has been made in the preparation of pure complex oligosaccharides through enzymatic,

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chemoenzymatic, or chemical methods.[14–17] Among others, the enzymatic[18] and the chemoenzymatic[19,20] approaches have provided an efficient access to GM2 and GM2-ceramide derivatives, respectively. After conjugation to a protein, the latter compound generated a carbohydrate-specific IgG response when injected in mice with Freund's complete adjuvant.[19] Furthermore, the chemical synthesis of an acetate-protected GM2 amino acid conjugate has been reported as a building block for the preparation of multivalent vaccine constructs.[21] It has been subsequently used for the stepwise assembly of a glycopeptidic structure carrying five or six different tumor-associated carbohydrate antigens.[22] However, it is important to note that these glycopeptides did not include any T cell epitopes and thus must be ultimately conjugated to a protein, resulting in hemi-synthetic vaccines similar to the KLH conjugates previously mentioned. The immunological properties of these multivalent GM2 conjugates have not been reported yet.

Although successful, the use of glycoconjugate hemi-synthetic vaccines has major limitations regarding their use in human.[23] These include 1) uncertainty in both composition and structure of the vaccine, 2) variable hapten density, 3) irrelevant Ab production against the carrier protein, and 4) carrier-induced epitopic suppression.[24] To overcome these drawbacks, a synthetic approach has been developed as an alternative. We and others have shown that fully synthetic neoglycopeptides are of great interest in anticancer immunotherapy.[25–30] Using this strategy, we have demonstrated that glycopeptides in which Tn clusters are linked to a $CD4^+$ T cell epitope peptide were very efficient in providing antitumor immunity in mice.[31] It is worth noting that there are still very few examples of synthetic vaccines so far as compared to the hemi-synthetic protein conjugates, and that synthetic ganglioside constructs have yet to be reported.

For all these reasons, there is a huge interest in designing new synthetic GM2 glycoconjugate vaccines and this undoubtedly relies on the availability of efficient synthetic methods. In this context, we focused our interest on the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition,[32,33] which is an archetypal example of click chemistry. Within a few years, this method has been established as one of the most efficient method for orthogonal chemoselective ligation, enabling the attachment of an alkyne functionalized molecule to an azide partner. It has found wide-ranging applications in bioconjugation, drug discovery and material science, among others.[34,35] Even though this method has been widely used for the synthesis of compounds with biomedical applications, very few examples of triazole-based immunogens have been described. Notably, Tn and sTn glycopeptides have been prepared using the cycloaddition chemistry. The peptide backbone was artificially designed as a model for a carrier protein, the ultimate goal being to prepare neoglycoproteins with the optimized method. However, only the synthesis of the model scaffold has been reported to date.[36] The synthesis of ovalbumin peptide–TLR ligand conjugates has also been described and the products were shown to stimulate a MHC class I restricted T cell hybridoma in vitro as efficiently as the peptide alone.[37] To our knowledge, this is the only example of immunological studies using a triazole-based antigen.

We have recently achieved the large-scale synthesis of GM2 in a form capable of being conjugated (alkyne derived) by using metabolically engineered *E. coli*.[38] Herein, we present the synthesis of GM2–peptide conjugates using the Huisgen cycloaddition, as well as their immunological evaluation. We show that bacteria-produced GM2 together with the click chemistry provide an efficient route to pure synthetic glycopeptide vaccines that can induce immune responses specific for human tumor cells.

Results and Discussion

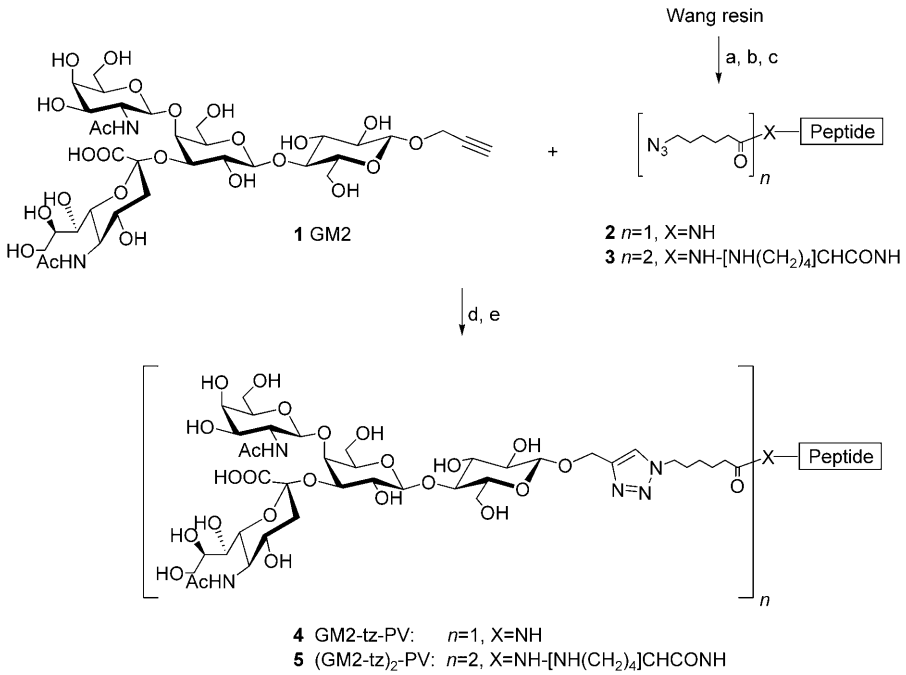
As carbohydrates are poorly immunogenic, we designed an immunogen capable of eliciting antibodies specific for the tetrasaccharidic portion of the GM2 ganglioside by incorporating a $CD4^+$ T cell epitope peptide from the VP1 protein of poliovirus type 1 (PV 103–115, KLFAVWKITYKDT).[39] Two carbohydrate–peptide conjugates were synthesized including one (GM2-tz-PV, **4**) or two ($(GM2\text{-}tz)_2$-PV, **5**) copies of the GM2 tetrasaccharide at the N terminus of the PV $CD4^+$ T cell peptide (Scheme 1).

The propargylated GM2 oligosaccharide **1** was prepared by high density culture of a recombinant *E. coli* strain engineered to biosynthesize and recycle nucleotide sugars, and to express appropriate glycosyltransferases.[38] Exogenous acceptors (*N*-acetyl neuraminic acid and propargyl-β-lactoside) were actively internalized into the cell cytoplasm by specific permeases before being glycosylated to provide the functionalized GM2 tetrasaccharide. Unlike chemical synthesis, this biotechnological process allowed a rapid and efficient gram-scale route to a conjugatable form of the GM2 oligosaccharide without resorting to expensive precursors or to enzyme isolation.

The PV peptide was assembled by conventional solid-phase synthesis. The chain was further extended at the N terminus with the 6-azidohexanoic acid to afford the monoazido PV **2**, which is suitable for the Huisgen cycloaddition. After cleavage from the resin and purification by reverse-phase (RP) HPLC, peptide **2** was subsequently attached to the GM2 alkyne derivative **1**. The reaction was monitored by analytical HPLC. The ligation was conducted in the presence of Cu^{II} and sodium ascorbate in H_2O/THF (1:1), and additional amounts of Cu^{II} and sodium ascorbate were added to force the reaction to completion. After two days, the crude compound was purified by RP-HPLC and the expected GM2-tz-PV **4** was isolated with excellent purity (59% yield, >99% purity).

A similar procedure was followed for the synthesis of $(GM2\text{-}tz)_2$-PV **5** except that an additional lysine residue was introduced at the N terminus of the PV peptide. 6-azidohexanoic acid was coupled simultaneously onto the $NH_2\alpha$ and $NH_2\varepsilon$ of the lysine residue, affording the diazido peptide **3**. Copper(I)-catalyzed cycloaddition of **3** to GM2 alkyne derivative **1** gave the di-GM2 glycoconjugate **5** after RP-HPLC purification in high purity (47% yield, >98.5% purity).

The GM2-triazolyl-biotin conjugate **6**, devoid of T helper epitope, was prepared in 59% yield by reacting **1** with *N*-(2-azido-



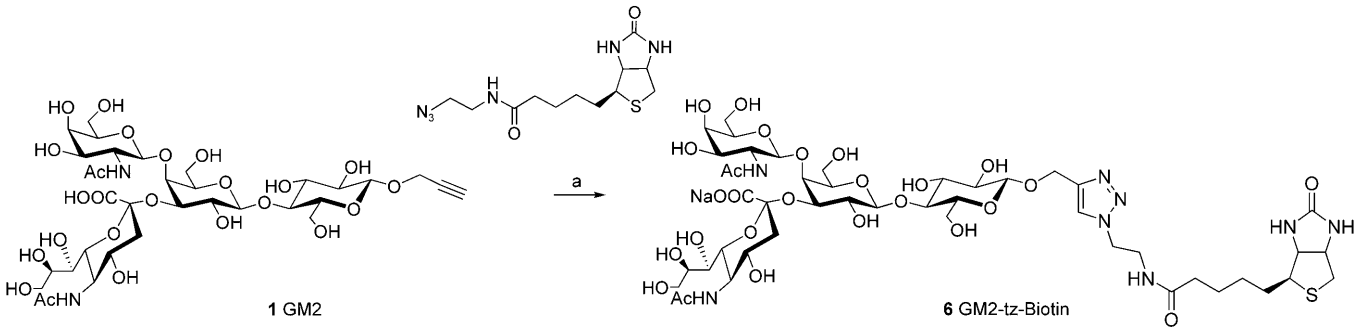
Scheme 1. Synthesis of the glycopeptides **4** and **5**. *Reagents and conditions*: a) Solid-phase peptide synthesis: Fmoc- and side chain-protected PV amino acids; Fmoc-Lys(Fmoc)-OH (when $n=2$); 6-azidohexanoic acid; b) TFA/TIS/H_2O (95:2.5:2.5); c) RP-HPLC; d) $CuSO_4$, sodium ascorbate, H_2O/THF (1:1); e) RP-HPLC.

ethyl)-biotinamide[40] in *t*BuOH/water (1:1) with the same catalytic system (Scheme 2).

We first assessed the functionality of the PV T cell epitope within the synthetic GM2–peptide conjugates **4** and **5** by testing their ability to be presented by dendritic cells and to stimulate T cells. As shown in Figure 1 a, compounds **4** and **5** can stimulate a specific T cell hybridoma as efficiently as the PV peptide devoid of GM2. We next tested the capacity of the synthetic constructs **4** and **5** to induce GM2 specific antibodies in vivo in a T cell dependent manner. Mice were immunized with **6**, devoid of T helper peptide, with compound **4** or with adjuvant alone, and sera from these mice were tested by ELISA for the recognition of GM2 and GM1 gangliosides (Figure 1 b and 1 c, respectively). In the absence of T helper peptide, compound **6** failed to induce any GM2-specific antibodies. In contrast, GM2-specific IgM (data not shown) and IgG antibodies were induced in mice immunized with compound **4** after two injections, however, the level of antibodies did not increase further after three injections (Figure 1 b). The specificity of GM2 antibodies induced is shown by the lack of recognition of asialo-GM2 and GM1 gangliosides by all sera (Figure 1 c and d).

We next compared the efficiency of GM2–PV neoglycopeptides **4** and **5** at inducing specific antibodies. As shown in Figure 1 d, in contrast to compound **4**, compound **5** failed to induce detectable level of either IgM or IgG antibodies in mice after four immunizations. To clearly determine that the antibodies elicited by the GM2–PV conjugates were able to recognize the native form of GM2 on tumor cells, we tested their binding to the human Jurkat tumor cell line and to melanoma cells expressing GM2 (Figure 2). Sera from mice immunized with compound **4** were able to recognize these human tumor cells, whereas sera derived from mice immunized with compound **5** did not (Figure 2 c and 2 d). The lack of native GM2 recognition by the sera of animals immunized with the di-GM2 compound (**5**) suggests that a neoglycosidic antigen has been generated without any cross reactivity for GM2. This latter result highlights the necessity of controlling the design and density of complex carbohydrate structures to appropriately mimic tumor-associated antigens.

To our knowledge, this is the first work reporting the induction of human tumor cell-specific antibodies after immunization with a synthetic glycopeptide, which is based on a ganglioside carbohydrate moiety produced in gram scale quantities by metabolically engineered bacteria. Their highly homogeneous and pure quality is very advantageous for consistent studies and safe clinical evaluation. Therefore, such glycopeptides



Scheme 2. Synthesis of the GM2-biotin conjugate **6**. *Reagents and conditions*: a) *t*BuOH/H_2O (1:1), $CuSO_4$, sodium ascorbate, RT, 40 h, 59%.

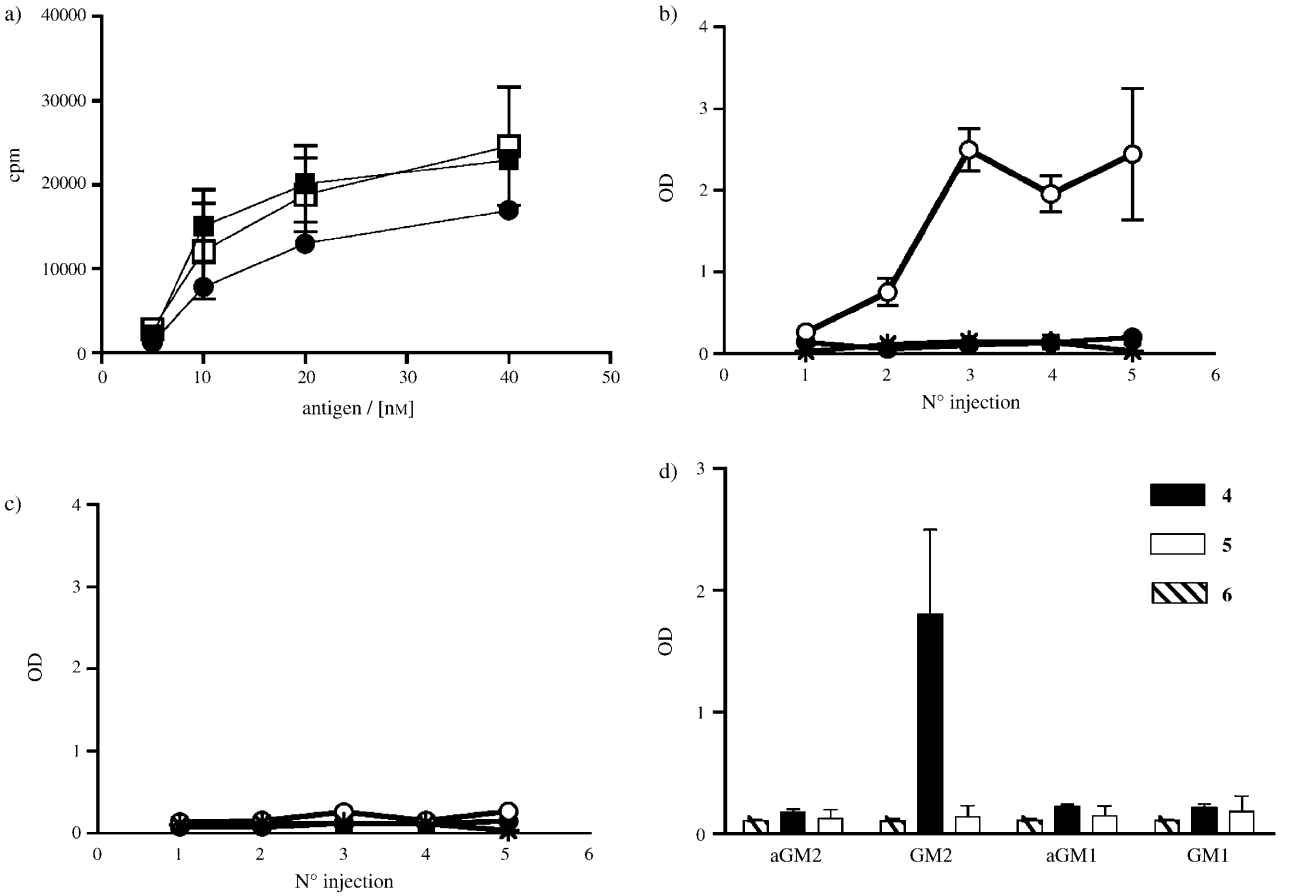


Figure 1. a) Immunogenicity of the GM2-PV glycopeptides **4** (●), **5** (■) and the PV peptide (KLFAVWKITYKDT) (□). Dendritic cells (1×10^5) were pulsed with indicated antigen and then used to stimulate PV-specific T cell hybridoma (1×10^5). Culture supernatants were collected at 24 h and tested for IL-2 content. BALB/c mice were immunized five times with 10 μg of **4** (○, $n=4$) or **6** (●, $n=4$) in adjuvant or with adjuvant alone (★, $n=2$), then mice were bled one week after immunization. Sera from these mice (1:100 dilution) were analyzed by ELISA for IgG antibodies against b) GM2 and c) GM1 gangliosides. d) Mice ($n=4$) were immunized 4 times at 3 week intervals with 10 μg of conjugate **4**, **5** or **6**. One week after the last injection, sera were collected and tested at 1:100 dilution by ELISA for IgG against aGM2, GM2, aGM1 and GM1 gangliosides. Results are expressed as the optical density (O.D.) measured at 492 nm.

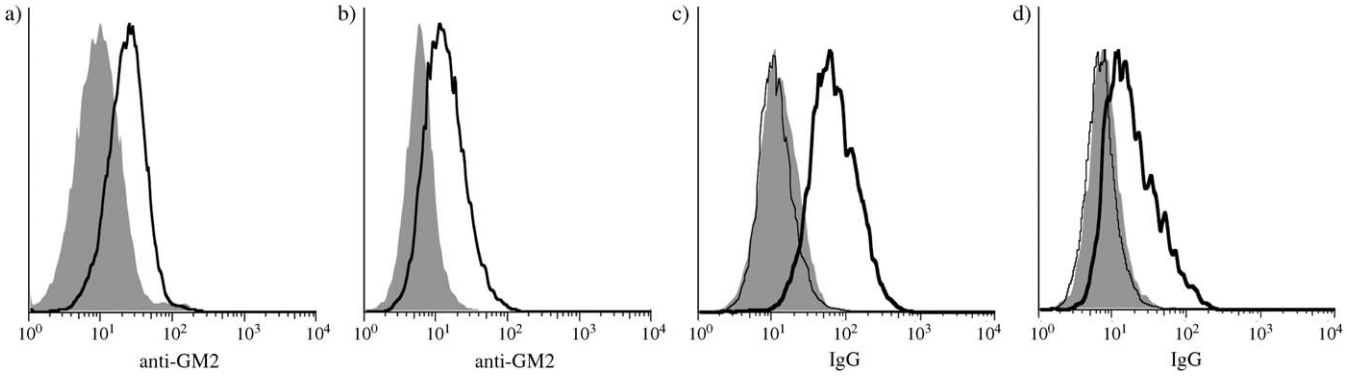


Figure 2. Analysis of tumor recognition by antibodies elicited by GM2-PV glycopeptides **4** and **5**. Staining of a) human Jurkat cells and b) NA8 melanoma cells by GM2-specific rabbit polyclonal antibodies (solid line) or control antibodies (filled grey). Pool of sera (1:100 dilution) obtained from mice ($n=4$) immunized with **4** (bold line) or **5** (thin line), as detailed in Figure 1 d, or control sera (filled grey), were analyzed by flow cytometry for the recognition of native GM2 on c) Jurkat cells and d) NA8 melanoma cells. The binding of antibodies to cells was revealed with phycoerythrin conjugated anti-mouse IgG antibodies.

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Experimental Section

General methods

The reagents used in peptide synthesis were purchased from AnaSpec (San Jose, USA) or Applied Biosytems (Courtabœuf, France). Piperidine and other chemicals were purchased from Sigma–Aldrich (St Quentin Fallavier, France). The intermediate peptides **2** and **3** were purified by RP-HPLC using a Perkin–Elmer pump system with a UV detector at 230 nm. The column was a C18 Kromasil (250× 10 mm, 5 μ, 200 Å, AIT, France). The final glycopeptides **4** and **5** were purified and analyzed by RP-HPLC using an Agilent 1100 instrument with a Diode Array detector at 230 nm. The column was a C18 Kromasil (250×4.6 mm, 5 μ, 100 Å, AIT, France). Elution was carried out with linear gradients of CH_3CN in 0.1% TFA buffer at a flow rate of 4 mL min^{-1} or 1 mL min^{-1} over 20 min.

All the peptides and glycopeptides were characterized by ES MS and amino acid analysis. The net peptide contents were determined by quantitative amino acid analysis using a Beckman 6300 analyzer after hydrolysis of the compounds with HCl (6 N) at 110 °C in sealed glass tubes for 20 h.

Mass spectra were recorded by electrospray in the positive mode on a Q-Tof Micro (Waters, Manchester, UK). The samples were dissolved at ~25 μM concentration in H_2O/CH_3CN (1:1) with 0.1% formic acid. NMR spectrum was recorded on a Brucker Avance 400 spectrometer. Chemical shifts are expressed as parts per million (ppm) relative to the solvent residual peak.

6-Azidohexanoic acid,[41] *N*-(2-azidoethyl)-biotinamide,[40] and propargyl-GM2 **1**[38] were prepared according to previously reported procedures.

Synthesis of glycopeptides GM2-tz-PV 4 and (GM2-tz)$_2$-PV 5

6-Azidohexanoic acid was prepared as previously described.[41] The PV peptide (KLFAVWKITYKDT) (0.033 mmol) was synthesized on a pre-loaded Wang resin with an Applied Biosystems ABI433 peptide synthesizer using Fmoc chemistry, NMP, and HATU and DIPEA as the coupling reagents. The Fmoc cleavages were carried out with piperidine (22% in NMP). Ultimately, the azido spacer arm N_3-$(CH_2)_5$-COOH was coupled manually using TBTU, HOBT and DIPEA as the coupling reagents and DMF as the solvent. The azido-peptide **2** was released from the solid support and simultaneously deprotected with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. After precipitation in Et_2O, the crude product was purified by RP-HPLC (32→45% CH_3CN) giving homogeneous **2** (34 mg, 59% overall). ESMS: *m/z* calcd for $C_{85}H_{130}N_{20}O_{20}$: 1751.977 $[M+H]^+$, found 1751.970. Amino acid analysis: Ala 1.11 (1), Asp 1.06 (1), Ile 0.93 (1), Leu 1.01 (1), Lys 2.96 (3), Phe 1 (1), Thr 1.90 (2), Tyr 0.96 (1), Val 0.94 (1).

A solution of GM2 **1**[38] (1.79 mg, 2 μmol) in H_2O (36 μL, 50 mg mL^{-1}) was added to a solution of peptide **2** (3.5 mg, 2 μmol) in H_2O (164 μL) and THF (215 μL). $CuSO_4{\cdot}H_2O$ (0.1 μmol, 3 mg mL^{-1} in water) was added, followed by a freshly prepared solution of sodium ascorbate (1 μmol, 20 mg mL^{-1} in water). The final solution was a 1:1 mixture of H_2O and THF. The mixture was stirred at RT and the reaction was monitored by RP-HPLC. After 24 h, the same amounts of **1**, $CuSO_4{\cdot}H_2O$ and sodium ascorbate solution were added. The reaction mixture was lyophilized after 48 h and the crude product was purified by RP-HPLC (25→50% CH_3CN). The main fractions were lyophilized to afford compound **4** (3.1 mg, 59% yield, >99% purity). ESMS: *m/z* calcd for $C_{119}H_{184}N_{22}O_{44}$: 2626.284 $[M+H]^+$, found 2626.283. Amino acid analysis: Ala 1.04 (1), Asp 1.05 (1), Ile 0.92 (1), Leu 1.02 (1), Lys 2.81 (3), Phe 1 (1), Thr 1.82 (2), Tyr 0.98 (1), Val 0.96 (1).

Compound **5** was synthesized following the procedure described above for **4** except that an additional lysine residue (Fmoc-Lys-(Fmoc)-OH) was introduced between the N terminus of the PV peptide and the azidohexanoic acid to dimerize the structure. Consequently, the number of equivalents of 6-azidohexanoic acid and GM2 **1** were doubled. The Fmoc protection was removed with 20% piperidine in DMF. The intermediate diazido peptide was purified by RP-HPLC (38→55% CH_3CN) giving homogeneous **3** (10 mg, 41% overall yield). ESMS: *m/z* calcd for $C_{97}H_{151}N_{25}O_{22}$: 2019.147 $[M+H]^+$, found 2019.135. Amino acid analysis: Ala 1 (1), Asp 1.0 (1), Ile 0.90 (1), Leu 0.96 (1), Lys 3.99 (4), Phe 0.98 (1), Thr 1.77 (1), Tyr 0.95 (1), Val 0.94 (1).

From compound **3** (1 mg, 0.5 μmol) the crude di-GM2 compound was obtained and purified by RP-HPLC (10→70% CH_3CN) to give conjugate **5** (0.88 mg, 47% isolated yield). ESMS: *m/z* calcd for $C_{165}H_{259}N_{29}O_{70}$: 3767.760 $[M+H]^+$, found 3767.756. Amino acid analysis: Ala 1 (1), Asp 1.2 (1), Ile 0.9 (1), Leu 1.0 (1), Lys 3.4 (4), Phe 0.9 (1), Thr 1.7 (1), Tyr 0.9 (1), Val 1.0 (1).

Synthesis of GM2-triazolyl-biotin conjugate 6

GM2 (**1**) (15 mg, 0.017 mmol) and *N*-(2-azidoethyl)-biotinamide[40] (5.3 mg, 0.017 mmol) were dissolved in *t*BuOH/H_2O (1:1, 1 mL). $CuSO_4$ (0.22 mg, 0.0014 mmol, 22 μL from a 10 mg mL^{-1} H_2O solution) and sodium ascorbate (2.8 mg, 0.014 mmol) were added and the mixture was allowed to stir at RT for 40 h before being concentrated in vacuo. The mixture was diluted in H_2O (1 mL) and purified through a Dowex 1×4–400 resin. Further purification by flash chromatography on silica gel (CH_3CN/H_2O, 8:2) afforded **6** (12 mg, 59%) as a white fluffy solid after freeze drying. ^{1}H NMR (400 MHz, D_2O) δ 8.1 (s, 1H, $H_{triazole}$), 5.02 (d, 1H, J=12.5 Hz), 4.90 (d, 1H, J=12.5 Hz), 4.76 (d, 1H, J=8.5 Hz, H-1^{GalNAc}), 4.63 (dd, 1H, J=5 Hz, J=8 Hz), 4.59 (m, 2H, H-1^{Glc}), 4.55 (d, 1H, J=8 Hz, H-1^{Gal}), 4.45 (dd, 1H, J=8 Hz, J=5 Hz), 4.18–3.61 (m, 25H), 3.51 (dd, 1H, J=2 Hz, J=10 Hz), 3.40–3.31 (m, 3H), 3.04 (dd, 1H, J=13 Hz, J=5 Hz), 2.82 (d, 1H, J=13 Hz), 2.69 (dd, 1H, J=12 Hz, J=5 Hz, H-3eq^{Neu}), 2.20 (t, 2H, J=7 Hz), 2.06 (s, 3H), 2.04 (s, 3H), 1.95 (t, 1H, J=12 Hz, H-3ax^{Neu}), 1.73–1.67 (m, 1H), 1.59–1.53 (m, 3H), 1.36–1.29 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 177.7, 175.7, 175.5, 174.8, 144.3, 126.2 ($CH_{triazole}$), 103.4 (C-1^{GalNAc}), 103.3 (C-1^{Gal}), 102.3 (C-2^{Neu}), 101.9 (C-1^{Glc}), 79.2, 77.8, 75.5, 75.4, 75.0, 74.7, 73.7, 73.3, 72.9, 72.0, 70.7, 69.4, 68.7, 68.4, 63.5, 62.7, 62.6, 61.8, 61.2, 60.9, 60.8, 55.9, 53.0, 52.6, 50.3, 40.4, 39.6, 37.6 (C-3^{Neu}), 36.0, 28.4, 28.3, 25.7, 23.3, 22.7 (2 × CH_3CO). HRMS: *m/z* calcd for $C_{46}H_{73}N_8O_{26}Na_2S$ $[M+Na]^+$ 1231.4152, found 1231.4174.

Analysis of T cell responses

The T cell antigenicity of GM2 glycopeptides was tested using bone marrow-derived dendritic cells incubated with PV, GM2-tz-PV **4** and (GM2-tz)$_2$-PV **5** for 24 h to stimulate a PV-specific T cell hybridoma response. Cell culture supernatants were frozen and IL-2 content was tested by following the proliferation of the IL-2 dependent CTL-L cell line through [^{3}H]thymidine incorporation. Results are expressed as counts min^{-1} (c.p.m.) detected under different conditions.

Analysis of antibody responses

The immunogenicity of the GM2 glycopeptides was assessed in BALB/c mice (CER Janvier, Le Genest St Ile, France). Animal studies were approved by the Institut Pasteur Safety Committee in accordance with French and European guidelines.

Mice were immunized (i.p.) every three weeks with **4**, **5** and **6** in alum supplemented with CpG1826 oligonucleotide (10 μg) (Proligo). In some experiments, mice were boosted 3 days after antigen administration with PolyI:C (50 μg, Invivogen). One week after immunization, sera were collected and tested for IgM and IgG antibodies by ELISA using asialo-GM1, asialo-GM2, GM1 and GM2 gangliosides purified from bovine brain (Sigma) and coated in MeOH at 0.5 μg. Serial dilutions of sera were performed and bound antibodies were revealed using goat anti-mouse IgG peroxidase conjugate (Sigma) and *o*-phenyldiamine/H_2O_2 substrate. Plates were read photometrically at 492 nm.

Sera were also tested at 1:100 dilution by flow cytometry for recognition of GM2-expressing human Jurkat tumor cell line and NA8 melanoma cells (obtained from Dr. P. Hubert, Institut Curie, France). GM2 expression by human cells was ascertained using polyclonal anti-GM2 rabbit antibodies (Abcam). Cells were first incubated for 30 min. with serial dilutions of sera at 4 °C in PBS containing 5% fetal calf serum and 0.05% sodium azide. Then, cells were incubated 30 min. with anti-mouse IgG or IgM conjugated to phycoerythrin (Caltag). Cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson) and analysis performed with Flowjo software (Tree Star Inc.). The data are shown as histograms corresponding to the fluorescence of cells incubated with the secondary reagent alone or together with sera and as mean of fluorescence.

HPLC profiles and NMR spectra for compounds **4** and **5**, and NMR spectra for compound **6** are available as Supporting Information.

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Keywords: antitumor agents · immunology · carbohydrates · glycopeptides · synthetic vaccines

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