Synthetic glucosylated peptide antigens of the bacterial protein adhesin of nontypeable Haemophilus influenzae to detect antibodies in Multiple Sclerosis

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Introduction

In previous studies, we demonstrated that antibodies in Multiple Sclerosis (MS), initially identified by a type I' β-turn glucopeptide, cross-reacted with the hyperglucosylated adhesin C-terminal fragment HMW1ct(1205-1536) of non-typeable Haemophilus influenzae [1]. The aberrant glucosylated moiety Asn(Glc), that is the common element between the glucopeptide and the bacterial adhesin protein, turned out to be the fundamental epitope to discriminate between sera of a subtype of MS patients and healthy individuals [2]. Since *N*-glucosylation is not present in eukaryotes as a post-translational modification, the hypothesis is that such a bacterial infection, can have an impact in triggering the autoimmune response in an MS patient subpopulation. The non-typeable Haemophilus influenzae (NTHi) bacterium was reported to express an glucosylated protein, the adhesin high molecular weight protein 1 (HMW1), which contains *N*-glucosyl moieties on asparagine residues in consensus *N*-glycosylation sites N-X-(S/T) (where X≠Pro) on β-turns. The HMW1ct(1205-1536), (Figure 1), was isolated and expressed as a 1:1:1 ratio of three *N*-glucosylated versions having 7, 8, and 9 glucose moieties on asparagines in N-X-(S/T) sequons. In HMW1ct(1205-1536) 12 glycosylation sequon sites are present, but only some of them appear to be located on the exposed loops of β-turns (sites 1, 2, 3, 5, 6, 7, 8 and 9). This is also in accordance with the computational model of the protein. (Figure 1).

Selected HMW1ct glucopeptides sequences

With the idea in mind to investigate the role of shorter peptides of hyperglucosylated HMW1ct adhesin protein in antibody recognition in MS, *N*-glucopeptides of adhesin bearing multiple *N*-Glc moieties were designed. In virtue of their spatial proximity and their optimal exposition on the same side of the protein, glucosylated asparagines could be preferentially targeted by anti-HMW1(Glc) antibodies. The selected fragments correspond to the peptide HMW1ct(1390-1403) including the glucosylation sites N^{1348}(Glc) and N^{1352}(Glc), and to the peptide HMW1ct(1345-1357) including the glucosylation sites N^{1393}(Glc) and N^{1398}(Glc). In particular the following adhesin peptide fragments specifically *N*-glucosylated were also synthesized by High-Temperature Fast-Stirring Solid-Phase Synthesis (HTFS-PS) (Table 1).

AHHHHHHVWTANSGALTTLAGSTIKGTESVTTSSQSGDIGGTISGGT
VEVKATESLTTQSNSKIKATTGEAN(1)VTSATGTIGGTISGNTVN(2)VT
ANAGDLTVGNGAEIN(3)ATEGAATLTTSSGKLTTEASSHITSAKGQVN
(4)LSAQDGSVAGSINAAN(5)VTLN(6)TTGTLTTVKGSNIN(7)ATSGTL
VINAKDAELNGAALGN(8)HTVVN(9)ATNAN(10)GSGSVIATTSSRV
N(11)ITGDLITINGLNIISKNGINTVLLKGVKIDVKYIQPGIASVDEVIEA
KRILEKVKDLSDEEREALAKLGVSAVRFIEPN(12)NTITVDTQNEFATR
PLSRIVISEGRACFSNSDGATVCVNIADNGR

Table 1. NTHi adhesin peptide analogs synthesized

Peptide	*N*-Glc moieties	Sequence
GP1 -[Asn^{1352}(Glc)]HMW1ct(1349-1357)	1 N-Glc	Ac-VTLN(Glc)TTGTL-NH_2
GP2- [Asn^{1348}(Glc), Asn^{1352}(Glc)]HMW1ct(1348-1357)	2 N-Glc	Ac-N(Glc)VTLN(Glc)TTGTL-NH_2
GP3-[Asn^{1348}(Glc), Asn^{1352}(Glc)]HMW1ct(1347-1357)	2 N-Glc	Ac-AN(Glc)VTLN(Glc)TTGTL-NH_2
GP4-[Asn^{1348}(Glc),Asn^{1352}(Glc)]HMW1ct(1345-1357)	2 N-Glc	Ac-NAAN(Glc)VTLN(Glc)TTGTL-NH_2
GP5-[Asn^{1398}(Glc)]HMW1ct(1390-1403)	1 N-Glc	Ac-TVVNATNAN(Glc)GSGSV-NH_2
GP6-[Asn^{1393}(Glc),Asn^{1398}(Glc)]HMW1ct(1390-1403)	2 N-Glc	Ac-TVVN(Glc)ATNAN(Glc)GSGSV- NH_2

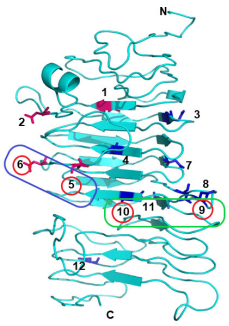
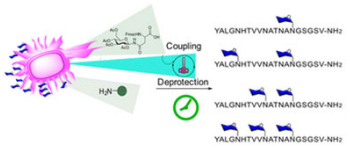


Figure 1. Structural model of the HMW1ct fragment calculated by ITASSER server [1] (right) and the corresponding amino acid sequence (left), with the 12 putative N-glucosylation sites.
The sequence HMW1ct(1345-1357) including the glucosylation sites N^{1393}(Glc) and N^{1398}(Glc) is highlighted in blue; HMW1ct(1390-1403) including the glucosylation sites N^{1348}(Glc) and N^{1352}(Glc), is highlighted in green.

Synthesis of a model glycopeptide bearing multiple copies of *N*-Glc moieties



Synthetic protocols for the synthesis of glycosylated peptides by high-temperature fast stirring-solid phase synthesis (HTFS-PS) [3]
All syntheses were carried out in the same reactor, using of TentaGel R RAM resin (100 mg, loading 0.18 mmol/g). All the steps in the process were performed at 90 °C with a stirring of 1200 rounds per minute (rpm).
Resin swelling. swelling in DMF for 30 min.
Fmoc-deprotection procedure. Washing with DMF (1 × 3 mL) for 30 sec. Fmoc-deprotection by 20% (v/v) piperidine/DMF in 30 sec Washing with DMF (1 × 3 mL) for 30 sec.
Coupling procedure. Coupling volume (3mL):1 ml of 7.2mM Fmoc-AA-OH in DMF was added to 1 mL, 6.6mM HATU in DMF and 1 mL of 14.4mM DIPEA in DMF. The reaction was stirred for 60 sec.
Fmoc-L-Asn[β-D-Glc(OAc)4]-OH were synthesized as previously described [4]
Final washing procedure. At the end of synthesis, the reactor was cooled to RT. Washings with DMF (1 × 3 mL), DCM (3 × 3 mL), MeOH (3 × 3 mL), and Et_2O (2 × 3 mL). The resin was dried under a vacuum.
Cleavage. Peptide cleavage was performed with a solution of TFA (5.5 mL), H_2O (0.3 mL), and TIPS (0.2 mL). The glycopeptides were precipitated in cold diethyl ether and lyophilized. After deacetylation of hydroxyl groups on glucose moieties, **GP1-6** peptides were purified by semi-preparative RP-HPLC and the glycopeptides purity was determined by HPLC and characterized by ESI-MS analysis. The analytical data are reported in details in Table 2.

Table 2. Analytical characterization of peptides

No	HPLC (Rt, min)[a]	ESI-MS (m/z) Found/calcd	HPLC Purity (%)	Yield (%)	Quantity (mg)
GP1	3.73	1123.00/1123.25[b]	95	1.5	2
GP2	3.40	1399.26/1399.50[b]	96	8.36	12
GP3	3.42	1470.38/1470.56[b]	96	6.0	8.8
GP4	3.35	1655.40/1655.76[b]	95	2	2
GP5	2.27	1494.29/1494.56[b]	95	1.54	2.5
GP6	1.43	1656.38/1656.71[b]	95	4.1	7

Peptides **GP 1-6** were purified by Reversed-Phase Flash Liquid Chromatography on a Biotage® Isolera™ (Biotage, Uppsala, Sweden) and Semi-preparative RP-HPLC on a Waters 600 chromatograph with solvent system A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). Analytical characterization of peptides **GP 1-6** was performed by analytical HPLC using a Waters Alliance instrument (model 2695, Milford, Massachusetts) coupled to a single quadrupole ESI-MS (Waters® ZQ Detector, Waters Milford, MA, USA) supplied with a BEH C18 (1.7 µm, 2.1× 50 mm) column at 35 °C, at 0.6 mL/min using solvent systems A (0.1% TFA in H_2O) and B (0.1% TFA in ACN). [a]Gradient elution was performed with a flow of 0.6 mL/min and started at 10% B, with a linear increase to 90% B in 5 min. [b]ESI-MS detected as $[M+H]^+$, $[M+2H]^{2+}$

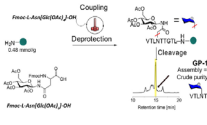


Figure. 2 HTFS-PS of **GP-1** on Rink Amide MBHA resin (0.48 mmol g^{-1} loading) solid support. Couplings were performed using 1.2 equiv. of all Fmoc-AAs, including Fmoc-L-Asn[Glc(OAc)4]-OH for 1 min at 90 °C using HATU/DIPEA as coupling system.

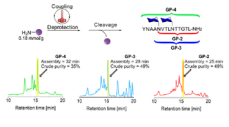


Figure 3. HTFS-PS of **GP-2**, **GP-3**, and **GP-4** on TentaGel R RAM resin (0.18 mmol g^{-1} loading). Coupling was performed using 1.2 equiv. of all Fmoc-AAs, including Fmoc-L-Asn[Glc(OAc)4]-OH and HATU/DIPEA coupling system for 1 min at 90 °C. HPLC analysis of the crude peptides were recorded at 220 nm.

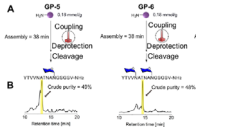


Figure 4. HTFS-PS of **GP-5** and **GP-6** on TentaGel R RAM resin (0.18 mmol g^{-1} loading) solid support (**A**). Couplings were performed using 1.2 equiv. of all Fmoc-AAs, including Fmoc-L-Asn[Glc(OAc)4]-OH and HATU/DIPEA as coupling system for 1 min at 90 °C. HPLC of crude (**B**) were recorded at 220 nm.

Results of the screening of Antibodies to the bacterial HMW1ct(Glc) peptides in Multiple Sclerosis patient sera

SP-ELISA:

- Peptides derived from NTHi adhesin bacterial protein HMW1ct(Glc) exhibited **no significant responses**, neither IgGs nor IgMs, in MS patient sera. We hypothesized that the sequences are **not long enough to coat** and recognize antibodies in the ELISA plate. In accordance with previous results, the antibody titre decreases in parallel with the length of the peptide immobilized [5].

COMING SOON **Inhibition Experiments in representative MS patient sera**

Conclusions

•HTFS-PS can become a prominent strategy to accelerate the synthesis of post-translationally modified peptides
•Antibody response is strictly influenced by the length of the immobilized peptide.

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