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GLYCOSYL DERIVATIVES OF NK_2 TACHYKININ RECEPTOR ANTAGONISTS

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Abstract: The influence of glycosylation on the structure-activity relationship of the tachykinin antagonist MEN 10376 and of its minimal active fragment MEN 10414 was investigated. The antagonist activity was preserved only when β-D-glucosylated Tyr, Ser and Asn were added to the *N*-terminal position of MEN 10376, while the modification of the side chain of the Tyr^2 induced a dramatic decrease in affinity.

Introduction

The carbohydrate moiety in glycoproteins plays a crucial role in protein biological functions. Glycosylation also affects conformation and stability of proteins.[1] Important effects of glycosylation have been proved also for peptides. Attractive features of glycopeptides as compared to their non-glycosylated parent analogues include increased serum half-life, broader spectrum of activity and a wider set of conformational variants, increased solubility and oral availability. For example, the introduction of sugar moieties on small hydrophobic peptides can produce the formation of compact, turn-folded conformations in aqueous solvents[2] and the glycosylation of some oligopeptide renin inhibitors increases their *in vivo* activity[3] and modifies the peptide clearance from rapid liver biliary clearance to slower urinary clearance.[4] A glycopeptide analogue of substance P (SP) has also been recently reported.[5] Among tachykinins, neurokinin A (NKA) has a great interest because activation of its receptor, termed NK_2, might be involved in disorders such as asthma, urinary incontinence and gut hypermotility.[6]

NK_2 antagonists are potentially useful to prevent bronchial hyper-reactivity and other pathological effects mediated by NKA. A series of selective NK_2 antagonists containing three D-Trp residues were previously reported.[7] We thought to apply the glycosylation approach to the NK_2 antagonists, MEN 10376 (H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2) and MEN 10414 (H-Asp-Tyr-D-Trp-Val-D-Trp-NH_2),[8] in order to investigate the effect of the carbohydrate moiety on their conformation and biological activity. This might also allow us to overcome the problem of the low solubility in aqueous media and, possibly, that of the short half-life of these very hydrophobic NK_2 peptide antagonists when administerd to living animals.[4]

We synthesised the *N*-, phenolic *O*- and aliphatic *O*-linked glycopeptides whose sequences are reported in Table 1.

Table 1. Structures of glycosyl derivatives of MEN 10376 and MEN 10414 NK_2 tachykinin receptor antagonists

Compound	Structure
MEN 11038	H-Asn(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2
MEN 10871	H-Asp-Tyr(Glc)-D-Trp-Val-D-Trp-NH_2
MEN 11201	H-Asp-Tyr(Glc)-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2
MEN 11202	H-Tyr(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2
MEN 11258	H-Ser(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2
MEN 11259	H-Ser(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-NH_2

Glc: β-D-glucopyranosyl residue.

The glycopeptide derivatives were tested on the isolated rabbit pulmonary artery (RPA) and hamster trachea (HT). These two preparations have been reported to contain two heterogeneous forms of the NK_2 receptor, on the basis of the affinity shown by certain NK_2 receptor-selective antagonists[7,9] such as our reference compound, MEN 10376 which shows high affinity for the rabbit NK_2 receptor.[10] On this basis, a further goal of the present study was to ascertain whether the introduction of the glucose moiety into MEN 10376 and MEN 10414 backbone could affect the selectivity of the novel compounds for these forms of the NK_2 receptor.

Results and Discussion

The syntheses of the new glycopeptides (Table 2) were performed by the solid phase methodology, using the known glycosylated building-blocks N^{α}-Fmoc-N'-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-Asn-OPfp,[13] N^{α}-Fmoc-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)Tyr-OPfp,[14] N^{α}-Fmoc-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-Ser-OPfp.[15] This is in fact the most versatile and general approach presently available for the preparation of a large variety of glycopeptides with well defined and predetermined structures. The Boc strategy is not compatible with glycopeptide synthesis because the interglycosidic bonds and the covalent linkage of sugar to the peptide are not stable in anhydrous HF or other strong acids used for the cleavage from the resin. The milder conditions used in the Fmoc strategy are much more compatible with the nature of the expected compound. For that reason sequential solid phase synthesis based on Fmoc strategy in combination with the use of suitable activated and glycosylated building-blocks has been the best method for the preparation of our model glycopeptides. Moreover, the use of pentafluorophenyl esters of Fmoc protected glycosylated amino acids, which are stable under the conditions used both for RP-HPLC and silica-gel chromatography, should allow a general application to the solid phase synthesis of all types of glycopeptides according to their chemical linkage. UV-monitoring of the Fmoc-cleavage was found to be important in order to avoid deletion sequences and impure final products.[13,16,17]

Table 2. Chemical data of the glycosyl derivatives of NK_2 tachykinin receptor antagonists[11]

Compound	Gradients and flow rates for semipreparative HPLC on Vydac column (ODS 218TP1010; 250 × 10 mm)[a]	Yield (%)	FAB-MS m/z, found $[M+H]^+$ (calcd.)	R_t (min)[a,b]
MEN 11038	20-60% B in 40 min (5 mL min^{-1})	7.5	1358 (1357.61)	8.64[c]
MEN 10871	25-60% B in 40 min (2.5 mL min^{-1})	8.7	929 (929.40)	11.5[d]
MEN 11201	20-60% B in 40 min (5 mL min^{-1})	9.4	1243.6 (1243.57)	7.59[e]
MEN 11202	30-70% B in 25 min (5 mL min^{-1})	5.3	1406.7 (1406.63)	7.56[f]
MEN 11258	Isocratic 30% B for 20 min, 30-60% B in 45.5 min (5 mL min^{-1})	7.5	1331 (1330.60)	11.54[g]
MEN 11259	25-60% B in 55 min (5 mL min^{-1})	12.3	1016.5 (1016.43)	10.44[h]

[a] Eluants: A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN); [b] Analytical HPLC on Vydac ODS 218TP54 (250 × 4 mm); [c] 30-60% B in 25 min (1 mL min^{-1}); [d] 25-60% B in 20 min (1 mL min^{-1}); [e] 30-70% B in 25 min (1 mL min^{-1}); [f] 30-70% B in 20 min (1 mL min^{-1}); [g] 25-60% B in 20 min (1 mL min^{-1}); [h] 25-60% B in 20 min (1 mL min^{-1}).

Reference compounds MEN 10376 and MEN 10414 showed competitive antagonism in both the RPA and HT preparations, and were more potent in the RPA (pA_2 = 8.1 and 6.3, respectively) than in the HT (pA_2 = 5.6 and 5.4, respectively). MEN 10376, in particular, exhibited a selectivity higher than 100 times for the NK_2 receptor of the RPA, as previously reported.[10] The glycosylated hepta- and octa-peptides MEN 11038, MEN 11201, MEN 11202 and MEN 11258 maintained the same pattern of selectivity being more potent antagonists in the RPA than in the HT (Table 3).[18] MEN 11201, with a glucosyl residue on the side chain of Tyr^2, was much weaker at NK_2 receptors of the RPA (pA_2 = 6.2) compared to the parent compound MEN 10376 (Table 3).

The lack in activity of MEN 10871 ($pA_2 < 5$) in the same preparation also confirms that the hydroxyl function of Tyr^2 is necessary for high affinity interaction.[19,20] In order to preserve the original side chains, the glycosylated amino acids were added to the *N*-terminal position of the antagonist sequence. As a result, MEN 11038, MEN 11202 and MEN 11258, with Asn(Glc), Tyr(Glc) and Ser(Glc) respectively in the *N*-terminal position, maintained a good antagonist activity in the RPA. On the contrary MEN 11259 showed a low potency in both preparations (Table 3).

Table 3. Antagonist activity at tachykinin NK_2 receptor of the glycosyl derivatives in RPA and HT[18]

	MEN 10376[a]	MEN 10414[b]	MEN 11038	MEN 10871	MEN 11201	MEN 11202	MEN 11258	MEN 11259
RPA	8.1 ± 0.1	6.3 ± 0.1	6.7 ± 0.1	≤ 5	6.2 ± 0.1	7.2 ± 0.1	7.3 ± 0.2	< 6
HT	5.6 ± 0.1	5.4 ± 0.2	IN	IN	IN	≤ 5	≤ 5	< 6

Biological values in the table are mean pK_B (negative logarithm of antagonist dissociation constant) ± S.E.M. of at least 9 determinations. Concentrations of test compounds higher than 10 μM were not tested because of the limited amounts synthesised. [a] Data are from Maggi et al. (ref. 10). [b] Data are from Quartara et al. (ref. 8). IN = inactive up to 10 μM.

These results indicate that the antagonist activity is preserved only when the glycosylated amino acids are added to the *N*-terminal position in the sequence and that the modification of the side-chain of Tyr^2 induces a dramatic decrease in the affinity for the NK_2 receptors. In the latter case, the decrease of activity might be attributed to the steric hindrance of the glycosyl moiety on the Tyr residue.

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11. All peptides were synthesised by the continuous flow solid phase synthesis on a semi-automatic apparatus (NovaSyn Gem Synthesizer) using Tentagel S RAM Fmoc resin (Rapp Polymere, Tübingen, Germany) for peptide amides (1 g, 0.24 mmol/g resin) following the Fmoc strategy using a 2.5-fold excess of Fmoc-amino acids with HOBt/TBTU/NMM activation in DMF. Acylation end points were determined by checking that the absorbance at 597 nm, due to the release of an anionic dye (acid Violet-17) from the cationic resin bound amino groups, did not change by more than 2 absorbance units over 10 min after a recirculation time of 30 min. Deprotection reactions (20% piperidine in DMF) were followed by monitoring at λ 365 nm the resulting dibenzofulvene-piperidine adduct. On completion of the synthesis the resin was washed with CH_2Cl_2, ether, and dried *in vacuo*. Peptides were cleaved and deprotected on the

side-chain at room temperature by using 96% TFA, 3% anisole and 1% ethanedithiole, unless otherwise stated. Deacetylation of the precursors of MEN 11038, 10871, 11201 and 11202, which was monitored by HPLC, was achieved by dissolving the crude material in dry MeOH and adding 0.1 M MeONa in MeOH until pH 12. The mixture was stirred at room temperature for 2 h, neutralised with solid CO_2 and concentrated. Debenzoylation of the precursors of MEN 11258 and 11259 following the procedure above described for deacetylation at pH = 10.5 was complete in 4 h. All the glycopeptides were purified by semi-preparative HPLC on a Gilson apparatus equipped with a UV detector, monitoring the eluate at 215 nm (254 nm or lower frequencies for the semi-preparative separations) and characterized by FAB-MS, analytical HPLC (Table I), amino acid analyses.[12] The final HPLC purity of the glycopeptides was always > 98%. Yields of purified glycopeptides were calculated as percentage of the theoretical yield, based on the substitution level of the resin.

12. Amino acid analyses with theoretical values in brackets. **MEN 11038**: Asn 0.89 (1), Asp 0.91 (1), Tyr 1.00 (1), Trp 2.91 (3), Val 0.88 (1), Lys 0.98 (1); **MEN 10871**: Asp 0.89 (1), Tyr 1.00 (1), Trp 1.95 (2), Val 0.87 (1); **MEN 11201**: Asp 0.89 (1), Tyr 1.00 (1), Trp 2.95 (3), Val 0.87 (1), Lys 0.98 (1); **MEN 11202**: Asp 0.87 (1), Tyr 2.00 (2), Trp 2.93 (3), Val 0.89 (1), Lys 0.97 (1); **MEN 11258**: Ser 0.98 (1), Asp 0.87 (1), Tyr 1.00 (1), Trp 2.93 (3), Val 0.89 (1), Lys 0.97 (1); **MEN 11259**: Ser 0.97 (1), Asp 0.87 (1), Tyr 1.00 (1), Trp 1.93 (2), Val 0.89 (1).
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18. Male albino rabbits (3.0-3.5 kg) and Syrian golden hamsters (100-120 g) were stunned and bled. Endothelium-denuded strips of rabbit pulmonary artery and rings of hamster trachea were excised and prepared for isometric tension recording in oxygenated (96% O_2 and 4% CO_2) Krebs solution in 5 ml organ baths, as previously described.[7] Concentration-response curves to the tachykinin agonist NKA were obtained in a cumulative manner, each concentration being added when the effect of the preceding one had reached a steady state. Preliminary experiments indicated lack of desensitisation of preparations to the cumulative administration of NKA. Tachykinin antagonists were assayed for their ability to inhibit the direct contractile response produced by NKA. At least three different concentrations of each antagonist were tested, in both bioassays. The competitivity of the interaction of each antagonist with the tachykinin NK_2 receptors was checked by the "Schild plot" method according to Arunlakshana and Schild.[21] Antagonists providing plots with linear regression lines and slopes not significantly different from unity were considered competitive. The affinity of competitive antagonists was expressed in terms of pK_B (negative logarithm of the antagonist dissociation constant), and, assuming a slope of -1, it was estimated

as the mean of the individual values obtained with the equation: pK_B = log [dose ratio –1] - log [antagonist concentration]. The values in the text, tables or figures are expressed as mean ± S.E.M.(standard error of the mean). Regression analysis of log concentration-effect curves was performed by the least squares method, considering linear such curves between 20 and 80% of the maximal response.

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