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Novel glycosylated VIP analogs: synthesis, biological activity, and metabolic stability

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Abstract: Vasoactive intestinal peptide (VIP) is a prominent neuropeptide, exhibiting a wide spectrum of biological activities in mammals. However, the clinical applications of VIP are mainly hampered because of its rapid degradation *in vivo*. Peptide glycosylation, a procedure frequently used to increase peptide resistance to proteolytic degradation and consequently increase peptide metabolic stability, has not been performed yet on VIP. The presence of three *N*-glycosylation sites on VIP receptor type 1 (VPAC1) was previously demonstrated. Therefore, glycosylation of the VIP ligand could potentially increase its receptor affinity because of glyco–glyco interactions between the ligand and the receptor. In order to enhance VIP's metabolic stability and to increase its ligand–receptor binding/activation, eight glycosylated VIP derivatives were successfully synthesized by the solid-phase procedure. Each VIP analog was monoglycosylated by a monosaccharide addition to one amino-acid residue along the sequence. Glycosylation did not affect the α-helical structure shown by the native VIP in organic environment. Few glycosylated VIP analogs displayed highly potent VPAC1 receptor binding and cAMP-induced activation; only 4–6 fold lower in comparison to the native VIP. Furthermore, the peptide analog glycosylated on Thr^{11} ([11Glyc]VIP) showed a significantly enhanced stability toward trypsin enzymatic degradation in comparison to VIP. Analysis of the degradation products of [11Glyc]VIP showed that differently from VIP, incubation of the peptide [11Glyc]VIP with trypsin resulted in no cleavage at the Arg^{12}–Leu^{13} peptide bond, suggesting that VIP glycosylation may lead to enhanced metabolic stability. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: VIP; cAMP; CD; enzymatic degradation; VPAC1; peptide glycosylation

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a prominent neuropeptide widely distributed in both the peripheral and the central nervous systems [1,2]. Effects of VIP are mediated through interaction with two receptors: VPAC1 and VPAC2 [3]. VIP may interact also with specific splice variants of pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (PAC1) [4–6]. VPAC1 and VPAC2 are preferentially coupled to Gαs protein that stimulates increases in adenylate cyclase [3]. Analyses including circular dichorism (CD), nuclear magnetic resonance (NMR), and theoretical calculations suggested that VIP secondary structure in ~50% organic environment is mostly helical with the existence of a central well-defined α-helix and a random coil structure at the *N*- and *C*-termini [7–9]. The study of Onoue *et al.*, [10] emphasizes the importance of the central α-helical structure in VIP by showing correlation between the α-helical content of VIP derivatives and their stomach relaxant activity. To date, there is no crystal structure of VIP or of the VIP ligand–receptor complex. Therefore, the structure–function relationships of VIP for interacting with VPAC1 were studied through Ala scans [9,11], binding studies to mutated receptor and through VIP probes containing photolabile residues in positions 6, 22, and 24 [12]. It was shown that VIP has diffuse pharmacophoric domains, with the amino acid residues important for biological activity being distributed along the whole 28-amino acid peptide chain [9,11].

Clinical applications of VIP have been suggested earlier for asthma, lung injury, diabetes, male impotence, a variety of tumors, and neurodegenerative diseases [2,13,14]. However, VIP is susceptible to rapid proteolytic degradation *in vivo* ($T_{1/2}$ of less than 1 min in humans plasma, Domschke *et al.*, [15]) resulting in low potency and short duration of action in clinical application. Therefore, it has been recognized that the

Abbreviations: DCM, dichloromethane; DIEA, *N*,*N*, di-isopropylethylamine; DMF, dimethylformamide; EDT, ethanedithiol; Fmoc, fluorenylmethoxy carbonyl; Gal, D-Galactopyranosyl; GlcNac, 2-Acetamido-2-deoxy-D-glucopyranosyl; HBTU, 2-(1 H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaflurophosphate; HOBT, 1-hydroxybenzotriazole; MBHA, [4(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamido-nor-leucyl-4-methyl-benzhydrylamine polystyrene]; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid; TIS, triisopropylsilane; For representing the alpha amino-acid residues that were glycosylated, the substituent is indicated in brackets before the amino acid three letter symbol.

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design of VIP analogs having high stability against digestive enzymes is a prerequisite for the optimal clinical use of VIP. Sugar addition to peptides (glycosylation) could potentially increase the peptide resistance against tissue peptidases and consequently increase the peptide half-life and raise its concentration in biological fluids [16–18]. Therefore, carbohydrate addition is repeatedly suggested for manipulating the metabolic stability of peptide drug leads. However, as far as we know, VIP glycosylation has not been performed to date.

Helospectin is a peptide that was purified from the venom of the Heloderma horridum lizard. Helospectin shows high homology to VIP and it binds with relatively high affinity to the VIP receptors [19]. Helospectin was shown to exist in a nonglycosylated form and in an *O*-glycosylated form with a *N*-acetylhexosamine-hexose motif. The glycosylated helospectin showed higher receptor affinity toward human VPAC1 receptor and higher potency in rat VPAC1 and VPAC2 receptor activation in comparison to the unglycosylated helospectin form [19].

The presence of three *N*-glycosylation sites (Asn^{58}, Asn^{69}, and Asn^{100}) in the *N*-terminal ectodomain of VPAC1 receptor was demonstrated by Couvineau *et al.*, [20]. Two glycosylation sites on Asn^{58} and Asn^{69} that are occupied by a 9-kDa *N*-linked carbohydrate were shown to play a mandatory role for the delivery of the VPAC1 receptor to the plasma membrane [20]. However, according to three-dimensional model of the *N*-terminal ectodomain of the human VPAC1, the two functionally important glycosylation sites on Asn^{58} and Asn^{69} are located at the surface of the structure of the *N*-terminal domain exposed toward the VIP ligand [21]. Therefore, VIP glycosylation could potentially yield chemical interactions between the glyco moiety on the VIP ligand and the glyco moiety on the VIP receptor that would yield higher receptor affinity and activation of the glycosylated VIP derivative in comparison to the native of VIP.

Here, we aim to enhance VIP's metabolic stability and to increase VIP ligand–receptor binding/activation through designing and synthesizing novel glycosylated VIP analogs. Since glycosylation could potentially increase VIP's resistance against tissue peptidases, glycosylated VIP analogs may have prolonged duration of action.

METHODS

Synthetic Procedure of the Preparation of Glycosylated VIP Analogs

Glycosylated VIP analogs were prepared by the solid-phase procedure on the Advanced ChemTech 348 Ω Peptide Synthesizer, starting with Rink Amide MBHA resin on a 0.06 mmol scale. The Fmoc-strategy and a single coupling with HBTU/HOBT/DIEA in NMP was used [18]. The deprotection of the Fmoc group was performed with 20% piperidine solution in NMP. To prevent the $\alpha-\beta$ shift at the Asp–Asn level from the addition of Asn^9 to the *N*-terminus, the deprotection step was carried out using a 6% solution of piperazine in DMF. The glycosylated amino-acid derivatives: Fmoc-[βGal(Ac)$_4$]Thr-OH [22], and Fmoc-[βGlcNAc(Ac)$_3$]Asn-OH [23] have been synthesized according to the literature. Fmoc-[βGal(Ac)$_4$]Ser-OH and Fmoc-[βGal(Ac)$_4$]Tyr-OH were obtained from INBIOS (Pozzuoli, Napoli, Italy). The final peptide resin was N^{α}-deprotected with piperazine in NMP, thoroughly washed with NMP and DCM, and dried. Cleavage from the resin and removal of the side-chain protecting groups were simultaneously achieved by treatment with a mixture of TFA/H_2O/TIS/EDT (94 : 2.5 : 2.5 : 1 by volume, 5 ml/100 mg of peptide resin, 2 h at room temperature). Yields of crude, glycosylated peptides were in the 80–85% range. Removal of the acetyl groups from the sugar moiety was achieved by dissolving the peptide (about 100 mg) in 5 ml of anhydrous methanol and adding 1% NaOMe (sodium methoxide) in methanol till pH 9; the reaction was quenched by addition of acetic acid, the solvent was evaporated *in vacuo* and the residue was lyophilized from water. The methionine residue (position 17 on the VIP sequence) is at least partially oxidized to methionine sulfoxide during the synthetic procedure. The methionine sulfoxide residue was reduced to methionine [24] by dissolving the peptide in TFA to give a 2.0 mM solution which was cooled to 0 °C. Ethyl methyl sulfide (20 eq) and NH_4I (20 eq) were added under vigorous stirring and the reaction was monitored by HPLC by removing samples from the reaction mixture. A saturated aqueous solution of ascorbic acid was added to the removed samples to quench the reduction and remove iodine. When the reaction was over, the mixture was further stirred for 3 h, then H_2O and carbon tetrachloride were added. The aqueous phase was washed with carbon tetrachloride, the solvent was removed *in vacuo*, and the residue was lyophilized from aqueous acetic acid. Analytical HPLC separations were performed on a Vydac C_{18} TP-104 column (250 × 4.6 mm, 10 μm, flow rate 1.5 ml/min). Eluants A (0.1% TFA in 90% aqueous CH_3CN) and B (aqueous 0.1% TFA) were used for preparing binary gradients (elution conditions: isocratic 10% A for 3 min, linear gradient 10–90% A in 30 min). Semipreparative HPLC separations (Vydac C_{18} 218 TP-1022 column, 250 × 22 mm, 10 μm, flow rate 15 ml/min) were performed on a Shimadzu series LC-6A chromatograph. Final yields, after HPLC purification, were in the 10–15% range. Molecular weight determinations were made by ESI-MS using an Applied Biosystems Mariner system 5220. For amino acid composition analysis, peptides were hydrolyzed in 6 N HCl at 100 °C for 24 h under vacuum, and the hydrolyzates were analyzed with a Dionex automatic amino-acid analyzer. Eight VIP analogs, that were monoglycosylated on different positions along the VIP sequence, were synthesized. In three of the VIP analogs, Met in position 17 was replaced by Nle (Table 1). *N*-Acetyl-D-glucosamine was β-*N*-glycosidically linked to the Asn residues in positions 9, 24, and 28 and D-galactose was β-*O*-glycosidically linked to Ser^2, Ser^{25}, Thr^7, Thr^{11}, and Tyr^{22}. In the following sections, we use the term Glyc adjacent to the residue number in order to mark the position of glycosylation. However, the peptide accurate nomenclatures are presented in Table 1.

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Table 1 The amino-acid sequence of the peptides and their mass spectra analysis are presented. The peptides were synthesized as *C*-terminal amides by solid-phase peptide methodology using the 9-fluorenylmethoxycarbonyl strategy. Fmoc glycosylated amino-acid derivatives were used as building blocks during the synthesis. Several glycosylated VIP analogs had Nle^{17} replacement instead of the Met^{17} in the native VIP. GlcNac, 2-acetamido-2-deoxy-D-glucopyranosyl. Gal, D-galactopyranosyl. The peptides were purified by Semipreparative HPLC separations (Vydac C_{18} 218 TP-1022 column, 250 × 22 mm, 10 μm, flow rate 15 ml/min) as described in the Section on Methods

Peptide name	Peptide sequence	MS found (calculated)
VIP (Vasoactive intestinal peptide)	$H_1SDAV_5FTDNY_{10}TRLRK\text{-}QM_{17}AVKKYLNSILN_{28}$	3327.7 (3325.9)
[Nle^{17}]VIP	[Nle^{17}]VIP	3310.6 (3308.0)
[2Glyc]VIP	[(βGal) Ser^2]-VIP	3488.8 (3487.9)
[7Glyc]VIP	[(βGal) Thr^7]-VIP	3489.8 (3487.9)
[9Glyc]VIP	[(βGlcNAc)Asn^9]-VIP	3529.8 (3529.0)
[11Glyc]VIP	[(βGal)Thr^{11}]-VIP	3488.7 (3487.9)
[22Glyc,Nle^{17}]VIP	[Nle^{17},(βGal)Tyr^{22}]-VIP	3472.0 (3469.9)
[24Glyc,Nle^{17}]VIP	[Nle^{17},(βGlcNAc)Asn^{24}]-VIP	3512.6 (3510.9)
[25Glyc]VIP	[(βGal)Ser^{25}]-VIP	3488.9 (3487.9)
[28Glyc,Nle^{17}]VIP	[Nle^{17},(βGlcNAc) Asn^{28}]-VIP	3513.1 (3510.9)

Cell Culture and Maintenance

The human colonic HT29 cells were routinely cultured in 75 cm^2 culture flasks in Dulbeco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose supplemented with 10% (V/V) fetal calf serum (FCS) and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, respectively) in a humidified atmosphere of air/CO_2 (95%/5%) at 37 °C [25]. Culture medium was replaced by fresh medium every 3 days. For subcultures, cells were harvested in Versene for 5 min at 37 °C [26,27].

Binding Assay on HT29 Cells

Binding studies were performed on intact HT29 cells according to previously reported conditions [25,26]. Briefly, cells (4×10^5 cells/well) were seeded on collagen-precoated 24 wells and cultured for 2 days. The cells were preincubated for 1 h at 4 °C and then incubated for 3 h at 4 °C in the presence of 50 pM ^{125}I-VIP (Amersham 2200 Ci/mmol) and increasing concentrations of VIP or VIP analogs in DMEM-50 mM HEPES (pH = 7.4) containing 1% bovine serum albumin (BSA), 0.1% bacitracin, and 150 μM phenylmethylsufonylfluoride (PMSF). Binding reactions were stopped by cooling the dishes on ice. Cells were rinsed once with 2 ml cold phosphate buffer saline (PBS) and lysed in 400 μl of 0.5 N NaOH. Radioactivity in cell lysates was quantified in a gamma counting system. Specific binding was calculated as the difference between the amount of ^{125}I-VIP bound in the absence (total binding) and presence of 1 μM unlabeled VIP (nonspecific binding).

Intracellular cAMP Accumulation

Cells (2×10^5) seeded in 24-well dishes were cultured for 3 days, after which their number was determined in three wells. Culture medium was then removed and cells were washed once with 500 μl of fresh medium and then equilibrated at 37 °C with 500 μl of medium containing 0.1 mM 3-isobutyl methyl xantine (IBMX) for 30 min. Cells were then incubated for 30 min at 37 °C after addition of the peptides. Control cells were treated with saline. The medium was then removed and the cAMP intracellular content was determined via the use of ELISA kit [26,27].

Circular Dichroism (CD) Studies

CD spectra were recorded on an AVIV-202 CD spectrometer (Lakewood, NJ, USA). Duplicate scans over a wavelength range of 190–260 nm were taken at ambient temperature. Peptides were dissolved in double distilled water (DDW) and trifluroethanol (TFE/DDW, 40/60, v/v) at a final concentration of 0.05 mM. A base line was recorded and subtracted after each spectrum. Elipticity is reported as the mean residue elipticity [ϕ] in deg-cm^2-$dmol^{-1}$. $[\phi] = [\phi]_{OBS}(MRW/10\,LC)$, where $[\phi]_{OBS}$ is the elipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide (molecular weight divided by the number of residues), *C* is the concentration of the sample in mg/ml, and *L* is the optical path length of the cell in centimeters [26,27].

Trypsin Catalyzed Cleavage of VIP and Glycosylated VIP Analogs

VIP and glycosylated VIP analogs were dissolved in 115 μl of 0.05M HEPES buffer, pH = 7.4, (100 μM final peptide concentration) and incubated with 10 μl of trypsin (1 μg/μl) at 37 °C. Ten microliters of 1 M HCl were added at a different time in order to stop the reaction. HPLC analysis allowed determination of percent of undigested peptide at each time. The peptides resulting from trypsin degradation were characterized by amino-acid analysis.

Data Analysis

Receptor binding and cAMP augmentation assay results are presented as the means of 2–4 different triplicate experiments.

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Enzymatic digestion results are presented as mean ± SD of four experiments ($P < 0.05$ by Mann–Whitney U test).

RESULTS

Synthesis of Novel Glycosylated VIP Analogs

Eight glycosylated VIP analogs were successfully prepared by solid-phase peptide synthesis using the Fmoc-strategy throughout peptide chain assembly (Table 1). Fmoc glycosylated amino-acid derivatives were used as building blocks during the synthesis. In order to achieve high receptor affinity, seven out of eight residues that were glycosylated here were previously defined as not involved in the VIP ligand receptor binding (only glycosylated Thr^7 in the VIP sequence was previously defined as involved in VIP receptor binding [9]). Mass spectra data of the HPLC-purified peptides is given in Table 1 and was in agreement with the expected mass.

Binding Experiments on HT29 Cells Expressing VPAC1 Receptor

HT29 cells expressing only the VPAC1 receptor were chosen for the analysis of binding of the various analogs [28]. VIP potently inhibited specific ^{125}I-VIP binding to HT29 cells. The IC_{50} (concentration of unlabeled peptide leading to half maximal inhibition of the ^{125}I-VIP specific binding) obtained for VIP ($IC_{50} = 2 \times 10^{-10}$ M, Table 2) is in good agreement with that reported by Lelievre for VIP on HT29 cells, namely, $IC_{50} = 6 \times 10^{-10}$ M [25]. [Nle^{17}]VIP inhibited the specific binding of ^{125}I-VIP with an equal IC_{50} to that of the native VIP (Table 2). This result is in agreement with previous studies [26,27,29]. All the glycosylated VIP analogs prepared so far in this study demonstrated reduction in VPAC1 receptor affinity ranging from 4 to 1700 fold lower as compared to the native VIP (Table 2). Generally, whereas VIP analogs glycosylated at the *C*-terminal region (Asn^{24}, Ser^{25}, or Asn^{28}) demonstrated only a slight reduction in receptor binding, 4–6 fold lower than the native VIP, the VIP analogs glycosylated at the *N*-terminal region (Ser^2, Thr^7, or Thr^{11}) demonstrated a more pronounced reduction in receptor affinity, 50–1700 fold lower than the native VIP. The VIP analogs [11Glyc]VIP and [7Glyc]VIP displayed the lowest VPAC1 receptor affinity, 1700 and 1000 fold lower than VIP, respectively (Table 2). The peptide [2Glyc]VIP showed a 20-fold higher affinity toward the VPAC1 receptor as compared with [7Glyc]VIP ($EC_{50} = 10^{-8}$ and 2×10^{-7}, respectively, Table 2). The VIP analogs [9Glyc]VIP and [22Glyc,Nle^{17}]VIP showed similar VPAC1 receptor affinity ($EC_{50} = 3 \times 10^{-9}$ and

Table 2 IC_{50} and EC_{50} values of VIP and glycosylated VIP analogs receptor binding and receptor-induced cAMP formation, respectively, on HT29 cells. In order to evaluate the peptide's receptor binding, cells (4×10^5 cells/well) were seeded on collagen-precoated 24 wells and cultured for 2 days. Incubations with radioligand (50 pM, ^{125}I-VIP) were performed at 4 °C during 180 min in the presence of increasing concentrations of unlabeled peptides. The IC_{50} values are the concentrations of unlabeled peptides that yielded half maximal inhibition of the ^{125}I-VIP specific binding on HT29 cells. In order to evaluate the peptide's receptor-induced cAMP formation, HT29 cells were seeded in 24-well culture plates 3 days before the experiment. Then, intact monolayer cultures were incubated for 30 min. at 37 °C in the presence of the indicated concentrations of VIP and VIP analogs in DMEM containing 0.1 mM IBMX. Intracellular cAMP levels were determined using commercially available ELISA kit. The EC_{50} values for elevation of cAMP levels are the concentrations of the peptides that resulted in 50% of the maximal response. Values are presented in molar (M) units. Data are the means of three–four independent triplicate experiments

Peptide name	IC_{50} (M)	Relative affinity	EC_{50} (M)	Relative potency
VIP	**2×10^{-10}**	1	**1×10^{-10}**	1
[Nle^{17}]VIP	**2×10^{-10}**	1	**1×10^{-10}**	1
[2Glyc]VIP	**10^{-8}**	0.02	**4×10^{-9}**	0.025
[7Glyc]VIP	**2×10^{-7}**	0.001	**2×10^{-7}**	0.0005
[9Glyc]VIP	**3×10^{-9}**	0.067	**9×10^{-10}**	0.11
[11Glyc]VIP	**3.5×10^{-7}**	0.00058	**6×10^{-8}**	0.0017
[22Glyc,Nle^{17}]VIP	**3.5×10^{-9}**	0.058	**10^{-9}**	0.1
[24Glyc,Nle^{17}]VIP	**8×10^{-10}**	0.25	**5×10^{-10}**	0.2
[25Glyc]VIP	**10^{-9}**	0.2	**6×10^{-10}**	0.167
[28Glyc,Nle^{17}]VIP	**1.2×10^{-9}**	0.17	**8.5×10^{-10}**	0.12

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3.5×10^{-9}, respectively, Table 2), approximately 16 fold lower than the native VIP.

Effects of VIP and Glycosylated VIP Analogs on Intracellular cAMP Levels

The EC_{50} value (concentration required to achieve 50% of the maximal effect) obtained by VIP ($EC_{50} = 1 \times 10^{-10}$ M, Table 2) correlates with its receptor binding ($IC_{50} = 2 \times 10^{-10}$ M, Table 2). VIP was highly efficient in increasing cAMP basal levels (~80-fold increase as compared to the control, data not shown). All the glycosylated VIP analogs were highly effective in increasing cAMP levels (~80-fold higher than control, data not shown) similar to the native VIP. Therefore, the glycosylated VIP analogs are VPAC1 receptor full-agonists. Generally, there was a good correlation between the EC_{50} for stimulating enzyme activity and the IC_{50} for inhibiting the ^{125}I-VIP specific receptor binding (Table 2). Thus, peptides [7Glyc]VIP and [11Glyc]VIP that showed the lowest receptor binding displayed the lowest potency in the cAMP-induced formation assay ($EC_{50} = 2 \times 10^{-7}$ and 6×10^{-8}, respectively, Table 2). Similarly, the peptide [2Glyc]VIP that had a 50-fold lower receptor affinity in comparison to VIP displayed similar reduction in the cAMP assay and was 40 fold less potent than VIP (Table 2). The VIP analogs glycosylated on residues Asn^{9}, Tyr^{22}, Asn^{24}, Ser^{25}, and Asn^{28} showed high potency in the cAMP augmentation assay, only 5–10 fold lower than the native VIP (Table 2). This result correlates with the high receptor affinity demonstrated by these peptides in the binding assay (Table 2).

Spectroscopic Study by CD

The CD spectrum of VIP in water shows one negative minimum at 198 nm (data not shown), indicative of a random coil structure [26,27]. The CD spectra of VIP in 40% TFE (Figure 1) revealed two negative minima, at 222 and 208 nm, indicating an α-helical structure. These results are in agreement with previous studies [7,12,30] that demonstrated that the central part of VIP adopts an α-helical structure in an organic environment. The CD curves of the glycosylated VIP analogs in water are very similar to that of VIP with one negative minimum around 198 nm (data not shown). Furthermore, the CD spectrum of the glycosylated VIP analogs in 40% TFE appeared highly similar to that of the VIP, with two minima, at 222 and 208 nm, indicating an α-helical structure (Figure 1, data presented for VIP and for the glycosylated VIP analogs that displayed the lowest and highest receptor affinity among the VIP analogs, [11Glyc]VIP and [24Glyc,Nle^{17}]VIP, respectively. Similar curves were demonstrated by all the glycosylated VIP analogs).

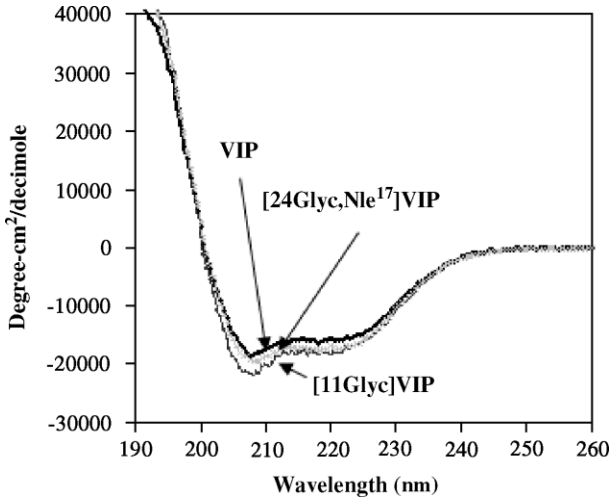

Figure 1 Circular dichorism spectra of VIP and two glycosylated VIP analogs ([11Glyc]VIP and [24Glyc,Nle^{17}]VIP) in 40% TFE. Similar spectra were revealed by all glycosylated VIP analogs. Elipticity is reported as the mean residue elipticity $[\phi]$ in deg-cm^2-d mol^{-1}. $[\phi] = [\phi]_{OBS}(MRW/10\,LC)$, where $[\phi]_{OBS}$ is the elipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide (molecular weight divided by the number of residues), C is the concentration of the sample in mg/ml, and L is the optical path length of the cell in cm.

Enzymatic Digestion by Trypsin

It has already been shown that glycosylation could reduce peptides susceptibility to proteolytic digestion and result in extended peptide activity [16,17]. The metabolic stability of the native VIP and the glycosylated VIP analogs was assessed by testing their resistance against trypsin enzymatic digestion. Monitoring by HPLC analysis during the incubation time showed that in the presence of trypsin, VIP was gradually digested and the $T_{1/2}$, time required to degrade 50% of the VIP, was 30 min (Figure 2). No significant degradation was observed by incubating the peptide in the absence of trypsin. Degradation curves were performed to all the glycosylated VIP analogs and the resulting $T_{1/2}$ of seven glycosylated VIP analogs was not significantly different from that of the native VIP. Only [11Glyc]VIP demonstrated a significantly increased stability toward trypsin degradation (Figure 2). After 45 min of incubation 48% of [11Glyc]VIP remained undigested whereas at the same time only 29% of the native VIP was still undigested (Figure 2). In order to elucidate the enhanced stability demonstrated by the [11Glyc]VIP in comparison with native VIP, an analysis of the major trypsin degradation products was performed. HPLC analysis of the crude mixture of VIP and trypsin following 30 min incubation showed the presence of five major peaks: one corresponding to the native VIP and the other four peaks corresponding to the VIP degradation products. Amino-acid analysis of the isolated peaks allowed identification of the following degradation products:

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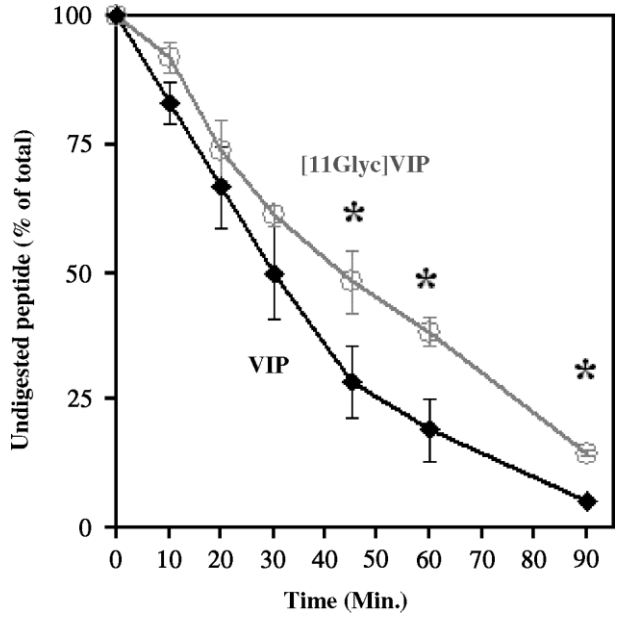

Figure 2 Enzymatic digestion of the native VIP and of the glycosylated VIP analog [11Glyc]VIP by trypsin. At the indicated period, the relative quantity of the intact native VIP and of undigested the VIP analog [11Glyc]VIP in the reaction mixture was determined by HPLC analysis. Each point represents the percentage (mean ± SD, $n = 4$) of undigested peptide. The peptide [11Glyc]VIP is significantly more resistant to trypsin degradation relative to the native VIP following 45, 60, and 90 min incubation ($P < 0.05$ by Mann–Whitney U test).

(i) VIP^{1-12}, (ii) VIP^{1-14}, (iii) VIP^{21-28}, and (iv) VIP^{15-28} (Figure 3). The interpretation of VIP's degradation products suggests that the cleavage of VIP occurred at the following sites: (i) the *C*-terminal side of Arg^{12}, (ii) the *C*-terminal side of Arg^{14}, and (iii) the *C*-terminal side of Lys^{20}. This is in agreement with the trypsin known activity to cleave peptides at the *C*-terminal side of Lys and Arg. Surprisingly only four major peaks were revealed at the same time point following incubation with trypsin of the peptide [11Glyc]VIP (Figure 3). One peak corresponded to the peptide [11Glyc]VIP and three other peaks corresponded to the degradation products of the peptide [11Glyc]VIP. Their retention times in the HPLC eluate were almost identical to those of the following VIP degradation products: (i) VIP^{1-14}, (ii) VIP^{21-28}, and (iii) VIP^{15-28} (Figure 3). It is noteworthy that in contrast to VIP, following incubation with trypsin of the peptide [11Glyc]VIP, the peptide bond Arg^{12}–Leu^{13} was not cleaved.

DISCUSSION

The study was designed to evaluate the impact of glycosylation on the VIP metabolic stability and receptor binding/activity. Results showed that few glycosylated VIP analogs maintained highly potent receptor binding and activation. Furthermore, a glycosylated VIP analog [11Glyc]VIP displayed significantly enhanced trypsin stability.

Eight monoglycosylated VIP derivatives were prepared by a monosaccharide addition to different residues along the VIP sequence. Though seven out of the eight glycosylations were performed on residues that are not involved in VIP receptor binding [9], all glycosylated VIP analogs displayed reduced (slight to drastic) receptor affinity in comparison with the native VIP. Since the glycosylated VIP analogs maintained an α-helical structure similar to the native VIP, it could be inferred that the glyco moieties did not substantially interfere with the peptide secondary structure. Therefore, it is suggested that the reduced receptor affinity demonstrated by the glycosylated VIP analogs could stem from steric or another hindrance of the glyco moieties to proximal residues that are essential to receptor

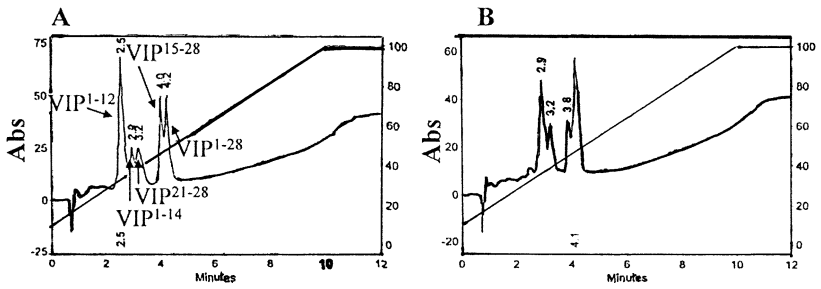

Figure 3 HPLC analysis of the crude mixture of VIP (left panel, A) and trypsin following 30 min incubation revealed five major peaks: one peak stands for the native VIP and four peaks correspond to VIP's degradation products. These peaks were examined by amino-acid analysis and the following degradation products were identified: (i) VIP^{1-12} (ii) VIP^{1-14}, (iii) VIP^{21-28}, and (iv) VIP^{15-28}. Surprisingly, HPLC analysis of the crude mixture of [11Glyc]VIP (right panel, B) and trypsin following 30 min incubation revealed only four major peaks: one peak stands for the peptide [11Glyc]VIP and three peaks correspond to [11Glyc]VIP degradation products. The three peaks that correspond to [11Glyc]VIP degradation products had retention times in the HPLC that were almost identical to the following VIP degradation products: (i) VIP^{1-14}, (ii) VIP^{21-28}, and (iii) VIP^{15-28}. Clearly, following the incubation of [11Glyc]VIP with trypsin there is no significant peak with retention time ~2.5 min (corresponding to the retention time of VIP^{1-12}) suggesting lack of significant cleavage at the *C*-terminal of Arg^{12}.

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binding, e.g. the glycosylation on Ser^{2} could interfere with the receptor interaction of the adjacent residues, His^{1} and Asp^{3} that are defined as important to induce potent receptor binding [9]. The rational to perform the glycosylation on residues that are not involved in receptor binding was to possibly strengthen weak ligand–receptor interactions and consequently enhance the receptor affinity. The attempt to strengthen weak VIP ligand–receptor interaction was successfully performed by Tams *et al.*, [31] who have generated a potent selective VPAC1 antagonist using this approach.

There was a good correlation between the receptor binding and the receptor-induced cAMP formation assays. Thus, all the glycosylated VIP analogs showed reduction in receptor-induced cAMP activation as compared to the native VIP. Nevertheless, it is noteworthy that the VIP analogs glycosylated on residues Asn^{9}, Tyr^{22}, Asn^{24}, Ser^{25}, and Asn^{28}, showed high potency in the cAMP augmentation assay, only 5–10 fold lower in comparison to the native VIP.

The possibility that either glycosylation of residues different from those modified in this study, or the use of different glyco moieties, for instance a disaccharide, would result in higher receptor affinity, could not be ruled out. Furthermore, the glycosylated VIP analogs were evaluated using the VPAC1 receptor that was very well characterized. Future studies will evaluate the effect of these analogs on the VPAC2 receptor and may yield different results.

The glycopeptide [11Glyc]VIP displayed significantly enhanced resistance against trypsin enzymatic digestion and in contrast with VIP, was not cleaved by trypsin in one of VIP's major cleavage site (Arg^{12}–Leu^{13}). The absence of trypsin cleavage at the *C*-terminal side of Arg^{12} could be rationalized by the steric hindrance of the glycan moiety (D-galactopyranosyl) attached to adjacent residue (Thr^{11}) that could interfere with the interaction of trypsin. It is noteworthy that the glycopeptide [11Glyc]VIP is a receptor full agonist. Rational design of VIP analogs di-glycosylated on two amino-acid residues adjacent to the two major tryptic sites of VIP (Arg^{12}–Leu^{13} and Arg^{14}–Lys^{15}) might drastically reduce the enzymatic cleavage at both these cleavage sites and consequently lead to a sharp increase in resistance toward trypsin degradation.

Deficiencies of VIP have been described in the airways in asthma patients, in the penises of impotent men and in the gastrointestinal (GI) tract in ulcerative colitis patients [14]. Administration of exogenous VIP in patients with such deficiencies could potentially be of therapeutic benefit. Even when a deficit of VIP is not a pathophysiological factor, it may be possible to treat the disease with VIP. For instance, the potent anti-inflammatory effects of VIP suggest its possible broad utility in controlling tissue inflammation. It is generally held that the rapid degradation of VIP is a major factor limiting the potential clinical applications of this peptide. Therefore, developing simplified, stable VIP analogs with high receptor affinity is highly desirable. Previously, various approaches were used in order to extend the duration of action of VIP following exogenous administration, e.g. cyclization of potential degradation sites [32], lyophilization [33,34] and liposome encapsulation of the peptide [35]. As far as we know, this is the first report describing the synthesis of glycosylated VIP derivatives. Glycosylation represents a novel and straightforward methodology to enhance the metabolic stability of VIP toward enzymatic degradation.

Over the last 20 years we have been studying the structure–function relationship of the peptide VIP and have been developing novel VIP analogs. We showed that substitution of the first six amino-acid residues of VIP with a partial six amino-acid sequence of neurotensin (6–11) yields a potent VIP antagonist [36]. Additionally, we prepared a VIP fatty derivative, termed SNV (stearyl-Nle^{17}-VIP), through the addition of a rather large moiety, stearic acid (18 carbons), to the *N*-terminal by an amide bond and the replacement of Met^{17} by Nle^{17}. SNV showed a 100-fold increase in potency as compared to VIP in promoting neuronal survival in a cAMP independent mechanism [37]. Furthermore, following the lyophilization approach, SNV clearly demonstrated superior skin penetration in comparison to VIP [34]. Recently, we evaluated the effect of multiplication of the *N*- or *C*-termini of VIP on the peptide receptor binding and activation potency [26,27]. A VIP analog with multiple *N*-terminal domains was shown to be slightly more potent toward VPAC1-related cAMP production as compared to [Nle^{17}]VIP [26]. Here, we generated a VIP glycosylated analog that demonstrated enhanced metabolic stability in comparison to the native VIP. We hypothesize that through combining the aforementioned chemical approaches (e.g. lyophilization and glycosylation) a VIP super-stable and super-potent analog could be generated.

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REFERENCES

1. Gozes I, Brenneman DE. VIP: molecular biology and neurobiological function. *Mol. Neurobiol.* 1989; **3**(4): 201–236.
2. Said SI. Vasoactive intestinal polypeptide biologic role in health and disease. *Trends Endocrinol. Metab.* 1991; **2**(3): 107–112.

3. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA. International union of pharmacology. XVIII. nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol. Rev.* 1998; **50**(2): 265–270.
4. Pisegna JR, Wank SA. Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. *Proc. Natl. Acad. Sci.* USA. 1993; **90**(13): 6345–6349.
5. Ashur-Fabian O, Giladi E, Brenneman DE, Gozes I. Identification of VIP/PACAP receptors on rat astrocytes using antisense digodeoxynucleotides. *J. Mol. Neurosci.* 1997; **9**: 211–222.
6. Pilzer I, Gozes I. VIP provides cellular protection through a specific splice variant of the PACAP receptor: a new neuroprotection target. *Peptides* 2006; **27**(11): 2867–2876.
7. Fry DC, Madison VS, Bolin DR, Greeley DN, Toome V, Wegrzynski BB. Solution structure of an analogue of vasoactive intestinal peptide as determined by two-dimensional NMR and circular dichroism spectroscopies and constrained molecular dynamics. *Biochemistry* 1989; **28**(6): 2399–2409.
8. Theriault Y, Boulanger Y, St-Pierre S. Structural determination of the vasoactive intestinal peptide by two-dimensional H-NMR spectroscopy. *Biopolymers* 1991; **31**(4): 459–464.
9. Nicole P, Lins L, Rouyer-Fessard C, Drouot C, Fulcrand P, Thomas A, Couvineau A, Martinez J, Brasseur R, Laburthe M. Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development of a highly selective VPAC1 receptor agonist. Alanine scanning and molecular modeling of the peptide. *J. Biol. Chem.* 2000; **275**(31): 24003–24012.
10. Onoue S, Matsumoto A, Nagano Y, Ohshima K, Ohmori Y, Yamada S, Kimura R, Yajima T, Kashimoto K. α-Helical structure in the C-terminus of vasoactive intestinal peptide: functional and structural consequences. *Eur. J. Pharmacol.* 2004; **485**: 307–316.
11. Igarashi H, Ito T, Hou W, Mantey SA, Pradhan TK, Ulrich CD II, Hocart SJ, Coy DH, Jensen RT. Elucidation of vasoactive intestinal peptide pharmacophore for VPAC(1) receptors in human, rat, and guinea pig. *J. Pharmacol. Exp. Ther.* 2002; **301**(1): 37–50.
12. Tan YV, Couvineau A, Murail S, Ceraudo E, Neumann JM, Lacapere JJ, Laburthe M. Peptide agonist docking in the N-terminal ectodomain of a class II G protein-coupled receptor, the VPAC1 receptor. Photoaffinity, NMR, and molecular modeling. *J. Biol. Chem.* 2006; **281**(18): 12792–12798.
13. Gozes I, Fridkin M, Hill JM, Brenneman DE. Pharmaceutical VIP. Prospects and problems. *Curr. Med. Chem.* 1999; **6**(11): 1019–1034.
14. Gozes I, Furman S. VIP and drug design [Review]. *Curr. Pharm. Des.* 2003; **9**(6): 483–494.
15. Domschke S, Domschke W, Bloom SR, Mitznegg P, Mitchell J, Lux G, Strunz U. Vasoactive intestinal peptide in man: Pharmacokinetics, metabolic and circulatory effects. *Gut* 1978; **19**: 1049–1053.
16. Negri L, Lattanzi R, Tabacco F, Scolaro B, Rocchi R. Glycodermorphins: opioid peptides with potent and prolonged analgesic activity and enhanced blood-brain barrier penetration. *Br. J. Pharmacol.* 1998; **124**(7): 1516–1522.
17. Negri L, Lattanzi R, Tabacco F, Orru L, Severini C, Scolaro B, Rocchi R. Dermorphin and deltorphin glycosylated analogues: synthesis and antinociceptive activity after systemic administration. *J. Med. Chem.* 1999; **42**(3): 400–404.
18. Gobbo M, Biondi L, Filira F, Gennaro R, Benincasa M, Scolaro B, Rocchi R. Antimicrobial peptides: synthesis and antibacterial activity of linear and cyclic drosocin and apidaecin 1b analogues. *J. Med. Chem.* 2002; **45**(20): 4494–4504.
19. Vandermeers-Piret MC, Vandermeers A, Gourlet P, Ali MH, Waelbroeck M, Robberecht P. Evidence that the lizard helospectin peptides are O-glycosylated. *Eur. J. Biochem.* 2000; **267**(14): 4556–4560.
20. Couvineau A, Fabre C, Gaudin P, Maoret JJ, Laburthe M. Mutagenesis of N-glycosylation sites in the human vasoactive intestinal peptide 1 receptor. Evidence that asparagine 58 or 69 is crucial for correct delivery of the receptor to plasma membrane. *Biochemistry* 1996; **35**(6): 1745–1752.
21. Lins L, Couvineau A, Rouyer-Fessard C, Nicole P, Maoret JJ, Benhamed M, Brasseur R, Thomas A, Laburthe M. The human VPAC1 receptor: three-dimensional model and mutagenesis of the N-terminal domain. *J. Biol. Chem.* 2001; **276**(13): 10153–10160.
22. Filira F, Biondi L, Scolaro B, Foffani MT, Mammi S, Peggion E, Rocchi R. Solid phase synthesis and conformation of sequential glycosylated polytripeptide sequences related to antifreeze glycoproteins. *Int. J. Biol. Macromol.* 1990; **12**: 41–50.
23. Biondi L, Filira F, Gobbo M, Scolaro B, Rocchi R. Synthesis of glycosylated tuftsins and tuftsin-containing IgG fragment undecapeptide. *Int. J. Pept. Protein Res.* 1991; **37**: 112–121.
24. Vilaseca M, Nicolas E, Capdevila F, Giralt E. Reduction of methionine sulfoxide with NH_4I/TFA; compatibility with peptides containing cysteine and aromatic residues. *Tetrahedron* 1988; **54**: 15273–15268.
25. Lelievre V, Meunier AC, Caigneaux E, Falcon J, Muller JM. Differential expression and function of PACAP and VIP receptors in four human colonic adenocarcinoma cell lines. *Cell. Signal.* 1998; **10**(1): 13–26.
26. Dangoor D, Rubinraut S, Fridkin M, Gozes I. Novel extended and branched N-terminal analogs of VIP. *Regul. Pept.* 2006; **137**(1–2): 42–49.
27. Dangoor D, Rubinraut S, Fridkin M, Gozes I. Novel Analogs of VIP with multiple C-terminal domains. *Peptides* 2007; **28**(9): 1622–1630.
28. Summers MA, O'Dorisio MS, Cox MO, Lara-Marquez M, Goetzl EJ. A lymphocyte-generated fragment of vasoactive intestinal peptide with VPAC1 agonist activity and VPAC2 antagonist effects. *J. Pharmacol. Exp. Ther.* 2003; **306**(2): 638–645.
29. Gourlet P, Rathe J, De Neef P, Cnudde J, Vandermeers-Piret MC, Waelbroeck M, Robberecht P. Interaction of lipophilic VIP derivatives with recombinant VIP1/PACAP and VIP2/PACAP receptors. *Eur. J. Pharmacol.* 1998; **354**(1): 105–111.
30. Fournier A, Saunders JK, Boulanger Y, St-Pierre SA. Conformational analysis of vasoactive intestinal peptide and related fragments. *Ann. N.Y. Acad. Sci.* 1988; **527**: 51–67.
31. Tams JW, Jorgensen RM, Holm A, Fahrenkrug J. Creation of a selective antagonist and agonist of the rat VPAC1 receptor using a combinatorial approach with VIP 6–23 as a template. *Mol. Pharmacol.* 2000; **58**(5): 1035–1041.
32. Bolin DR, Cottrell J, Garippa R, O'Neill N, Simko B, O'Donnell M. Structure-activity studies of vasoactive intestinal peptide (VIP): cyclic disulfide analogs. *Int. J. Pept. Protein Res.* 1993; **41**(2): 124–132.
33. Gozes I, Fridkin M. A fatty neuropeptide. *J. Clin. Invest.* 1992; **90**: 810–814.
34. Gozes I, Reshef A, Salah D, Rubinraut S, Fridkin M. Stearyl-norleucine-vasoactive intestinal peptide (VIP): a novel VIP analog for noninvasive impotence treatment. *Endocrinology* 1994; **134**(5): 2121–2125.
35. Gololobov G, Noda Y, Sherman S, Rubinstein I, Baranowska-Kortylewicz J, Paul S. Stabilization of vasoactive intestinal peptide by lipids. *J. Pharmacol. Exp. Ther.* 1998; **285**(2): 753–758.
36. Gozes I, McCune SK, Jacobson L, Warren D, Moody TW, Fridkin M, Brenneman DE. An antagonist to vasoactive intestinal peptide affects cellular functions in the central nervous system. *J. Pharmacol. Exp. Ther.* 1991; **257**(3): 959–966.
37. Gozes I, Lilling G, Glazer R, Ticher A, Ashkenazi IE, Davidson A, Rubinraut S, Fridkin M, Brenneman DE. Superactive lipophilic peptides discriminate multiple vasoactive intestinal peptide receptors. *J. Pharmacol. Exp. Ther.* 1995; **273**(1): 161.