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Novel extended and branched N-terminal analogs of VIP

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Abstract

The effects of vasoactive intestinal peptide (VIP) are primarily mediated through VPAC1 and VPAC2, receptors that are preferentially coupled to adenylate cyclase activation. As a large majority of the potent VIP antagonists have modifications in the N-terminal domain of the peptide, the effect of multiplication of this domain on VIP was examined with the aim of possibly amplifying peptide-receptor (VPAC1) activation. Several VIP analogs were designed and synthesized, each carrying multiplication of the N-terminal domain that was obtained by either linear tandem extension or by parallel branching. Circular dichorism (CD) analysis revealed that these extended/branched peptides maintained an α helical structure in organic environment, similar to VIP. A specific branched VIP analog was found to be slightly more potent towards VPAC1-related cAMP production as compared to VIP. This analog could have potential therapeutic value in several disorders, similar to VIP. Two branched N-terminal VIP sequences demonstrated superior receptor binding and activation as compared to two N-terminals in tandem. The results suggest that correct alignment of the VIP N-terminal region is important for receptor binding and activation. However, increased receptor binding was not directly associated with increased cAMP production suggesting steric dynamic interactions.

Keywords: VIP; VPAC1; cAMP; CD; HT29 cells; Peptides; Receptor binding

1. Introduction

Vasoactive intestinal peptide (VIP) is a prominent neuropeptide widely distributed in both the peripheral and the central nervous systems. VIP exhibits a large spectrum of biological actions in mammals [1–3]. VIP-containing nerves and VIP mediated effects have been described in the digestive tract, cardiovascular system, airways, reproductive system, immune system, endocrine glands and the brain [3–5]. VIP has also been characterized as a growth regulator of tumor cells and of the developing embryo. In the newborn rodent, VIP was suggested to regulate brain development [1]. Clinical applications of VIP and VIP analogs have been suggested for male impotence, asthma, lung injury, a variety of tumors and neurodegenerative diseases [1,3,6]. Effects of VIP are mediated through high affinity interaction with two receptors: VPAC1 and VPAC2 [7]. VIP may interact also (low affinity interaction) with the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (PAC1) [8,9]. VPAC1 and VPAC2 are preferentially coupled to the Gαs protein that stimulates increases in adenylate cyclase [7]. These receptors, together with receptors for VIP-related peptides (e.g. PACAP, secretin, glucagon), clearly constitute a unique subfamily within the superfamily of G protein-coupled receptors, which is referred to as class II G protein-coupled receptors [10]. Class II family of receptors for peptides share several common properties including: 1] seven membrane spanning domains, 2] large N-terminal extracellular domains containing highly conserved cysteine residues, 3] N-terminal leader sequences, and 4] complex gene organization with many introns [11]. To date, there is no crystal structure of VIP or of the VIP receptor complex. 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 [12–14].

The structure–function relationships of VIP for interacting with VPAC1 was studied through Ala scans [12,15,16], complete

Abbreviations: CD, circular dichorism; VIP, vasoactive intestinal peptide; VPAC, VIP receptor.

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Table 1
Amino-acid sequence of the peptides and their mass spectra analysis

Peptide name	Abbreviation	Peptide sequence	MH^+ found (calculated)
[Nle^{17}] vasoactive intestinal peptide	[Nle^{17}]VIP	$H_1SDAV_5FTDNY_{10}TRLRKQ[Nle]_{17}AVKKYLNSILN_{28}$	3310.6 (3308.0)
Extended 1	EX-1	HSDAVF-(β-Ala)$_0$-[(Nle^{17})$VIP_{1–28}$]	4036.7 (4035)
Extended 2	EX-2	FVADSH-(β-Ala) $_0$-[(Nle^{17})$VIP_{1–28}$]	4037 (4035)
Branched 1	BR-1	$(HSDAVFTD)_2$Lys-[(Nle^{17})$VIP_{10–28}$]	4196.3 (4194.4)
Branched 2	BR-2	$(HSDAVFT)_2$Lys-[(Nle^{17})$VIP_{9–28}$]	4080.3 (4079)
Branched 3	BR-3	$(HSDAVFTDNY)_2$Lys-[(Nle^{17})$VIP_{12–28}$]	4486.5 (4485.1)
Branched 5	BR-5	[$(HSDAVFT)_2$Lys]$_2$Lys-[(Nle^{17})$VIP_{9–28}$]	5854.5 (5850)
Branched 6	BR-6	$(HSDAVF)_2$Lys-[(Nle^{17})$VIP_{8–28}$]	3993.4 (3992)
Branched 7	BR-7	$(HSDAV)_2$Lys-[(Nle^{17})$VIP_{7–28}$]	3795.8 (3800)

All VIP analogs had Nle^{17} replacement instead of Met^{17} in the native VIP. The peptides were synthesized as C-terminal amides by solid-phase peptide methodology using the 9-fluorenylmethoxycarbonyl strategy. Crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Vydac Protein/Peptide 218TP C-18 column (10×250 mm, 12 μm bead size; Vydac, Hesperia, CA, USA), employing a binary gradient formed from 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B). The chromatographic run (flow rate 10 ml/min) started with 10% of solution B in solution A, kept constant for 10 min, followed by a gradient increase of solution B from 10 to 100% over additional 50 min. The isolated peptides were subjected to analytical RP-HPLC under similar conditions as above to confirm purity.

amino-acid substitutional analysis [17] and binding studies to the mutated receptor [11]. However, the effect of "large-scale" modifications of VIP on receptor binding and activation has not been examined. In order to elucidate the physiological role of VIP, several antagonists have been developed. A large proportion of potent VIP antagonists have modifications in the N-terminal domain of the peptide, including deletion of the first five [18] or eight amino acids in the sequence of VIP [19], and substitution of the first six amino-acid residues with a partial six amino-acid sequence of neurotensin (6–11) [20,21]. Thus, it is likely that the N-terminal part of VIP is associated with peptide activity. This suggestion is in accordance with the study of Juarranz et al. [22], which suggested that the N-terminal domain of VIP is responsible for the activation of the receptor through interaction with the transmembrane receptor core. Linear extensions and branching are two common methodologies that are used to generate peptide derivatives. Previously, we showed increased biological activity of a VIP derivative that included a linear extension of a lipophilic moiety at the N-terminal of the peptide [23]. Peptide branching could be performed through synthesizing peptides on a polylysine core. This methodology is widely used for the preparation of multiple antigenic peptides (MAP). Bracci et al. [24] demonstrated that a tetrabranched MAP form of α-bungarotoxin binding peptide while having a similar IC_{50} as compared with that of the monomeric peptide, was at least 100 times more active *in vivo*.

The aim of this study was to design, synthesize and discover VIP analogues that exhibit increased receptor activation. As the N-terminal of VIP is associated with peptide activity, we tested the hypothesis that multiplication (linear or branched) of the N-terminal site may lead to enhanced peptide-receptor association and/or amplified receptor activation.

2. Materials and methods

2.1. Peptide synthesis

Peptides were prepared as C-terminal amides by conventional solid-phase peptide synthesis on rink amide resin, using an ABIMED AMS-422 automated solid-phase multiple peptide synthesizer (Langenfeld, Germany). The 9-fluorenylmethoxycarbonyl (Fmoc) strategy was used throughout the peptide chain assembly [25]. Crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Vydac Protein/Peptide 218TP C-18 column (10×250 mm, 12 μm bead size; Vydac, Hesperia, CA, USA), employing a binary gradient formed from 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B). The chromatographic run (flow rate 10 ml/min) started with 10% of solution B in solution A, kept constant for 10 min, followed by a gradient increase of solution B 10 to 100% over additional 50 min. The isolated peptides were subjected to analytical RP-HPLC under similar conditions as above to confirm their purity. Mass spectrometry was performed on a Micromass Platform LCZ 4000 (Manchester, UK) utilizing electron spray ionization method. 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.

VIP analogs that were designed to have the N-terminal of the peptide (first six amino acids) attached via a β-Ala residue through the amino terminal of VIP to the whole sequence of VIP (Table 1) were termed extended. Two extended VIP analogs were created that differed in the orientation of the first six amino acids of VIP. One analog, termed EX-1, had a linear extension with the duplicate segment ($VIP_{1–6}$) at the N-terminal. In the second peptide, termed EX-2, the extension was made up of the same residues in the reversed order, $VIP_{6–1}$, (Table 1). Branched N-terminal VIP analogs were prepared by an amino-acid replacement with the bi-functional amino-acid Lysine at the branching point. The different analogs included replacement of either Phe^6, or Thr^7, or Asp^8, or Asn^9, or Thr^{11} on the linear VIP peptide. After the coupling of the Lys residue to the growing VIP peptide chain, twofold equivalents of the following amino acids were added (one by one). It should be taken into consideration that chemical peptide synthesis on solid-phase progresses from C-terminal to N-terminal. The branching point Lys having two free amino groups (located α and ε to the carboxylic group) allowed parallel extensions to result in a branched peptide with two identical N-terminal sequences (Table 1).

Table 2
Amino-acid composition of synthesized peptides

Peptide	Asp/*n*	Ser	Glu/*n*	His	Arg	Thr	Ala	Tyr	Val	Lys	Ile	Leu	Nle	Phe	β-Ala
[Nle^{17}]VIP	5(4.93)	2(1.70)	1(1.13)	1(0.86)	2(2.15)	2(1.93)	2(1.98)	2(2.01)	2(1.89)	3(3.40)	1(1.08)	3(3.08)	1(1.05)	1(0.85)	
EX-1	6(5.83)	3(2.84)	1(1.12)	2(1.97)	2(2.23)	2(2.07)	3(3.11)	2(1.98)	3(3.01)	3(3.20)	1(1.02)	3(2.98)	1(0.94)	2(1.99)	1(1.00)
EX-2	6(5.90)	3(2.74)	1(1.09)	2(1.92)	2(2.26)	2(2.09)	3(2.96)	2(1.99)	3(3.00)	3(3.25)	1(1.05)	3(3.07)	1(0.97)	2(1.90)	1(1.00)
BR-1	6(5.72)	3(2.64)	1(1.04)	2(1.83)	2(2.10)	3(2.90)	3(3.09)	2(1.76)	3(2.88)	4(3.89)	1(1.00)	3(2.94)	1(0.96)	2(1.85)	
BR-2	5(4.91)	3(2.65)	1(1.04)	2(1.86)	2(2.16)	3(2.92)	3(3.07)	2(1.73)	3(2.92)	4(4.03)	1(1.03)	3(2.99)	1(0.99)	2(1.90)	
BR-3	8(7.57)	3(2.66)	1(1.04)	2(1.81)	2(2.10)	2(1.90)	3(3.01)	3(2.60)	3(2.89)	4(3.97)	1(1.02)	3(2.98)	1(0.96)	1(.090)	
BR-5	7(6.81)	4(4.27)	1(1.06)	4(3.52)	2(2.16)	5(4.81)	5(5.16)	2(1.90)	5(4.89)	6(6.00)	1(1.03)	3(3.02)	1(0.99)	4(3.78)	
BR-6	6(5.83)	3(2.49)	1(1.18)	2(1.76)	2(2.20)	1(1.01)	3(2.89)	2(1.86)	3(2.77)	4(4.29)	1(1.08)	3(3.11)	1(1.02)	2(1.72)	
BR-7	6(5.79)	3(2.58)	1(1.13)	2(1.83)	2(1.87)	2(1.88)	3(2.97)	2(1.72)	3(2.77)	4(4.23)	1(1.08)	3(3.14)	1(0.95)		

Calculated amino-acid ratios are presented as compared to the ratios measured. 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.

2.2. Cell culture and maintenance

The human colonic HT-29 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 [26]. Culture medium was replaced by fresh medium every three days. For subcultures, cells were harvested in Versene for 5 min at 37 °C.

2.3. Binding assay on HT 29 cells

Binding studies were performed on intact HT 29 cells according to previously reported conditions [21,27]. Briefly, cells (4×10^5 cells/well) were seeded in 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 (non-specific binding).

Table 3
IC_{50} values of binding of [Nle^{17}]VIP and VIP analogs on HT 29 cells

Peptide	IC_{50} (M)	Relative affinity	EC_{50} (M)	Relative potency
[Nle^{17}]VIP	7×10^{-10}	1	1×10^{-10}	1
EX-1	4.5×10^{-7}	0.0015	5.5×10^{-8}	0.0018
EX-2	5×10^{-7}	0.0014	3×10^{-8}	0.0033
BR-1	1×10^{-8}	0.07	4.3×10^{-11}	2.3
BR-2	4.5×10^{-9}	0.1556	1.8×10^{-9}	0.055
BR-3	1×10^{-7}	0.007	1×10^{-8}	0.01
BR-5	$>10^{-6}$	<0.0007	$>10^{-7}$	<0.001
BR-6	$>10^{-6}$	<0.0007	$>10^{-7}$	<0.001
BR-7	$>10^{-6}$	<0.0007	$>10^{-7}$	<0.001

The IC_{50} values were established from inhibition curves of tracer binding ^{125}I-VIP on HT 29 cells. 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.

2.4. 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 (Amersham).

2.5. Circular dichorism (CD) studies

CD spectra were recorded on an AVIV-202 circular dichorism spectrometer (Lakewood, NJ, USA). Duplicate scans over a wavelength range of 190–260 nm were taken at ambient temperature.

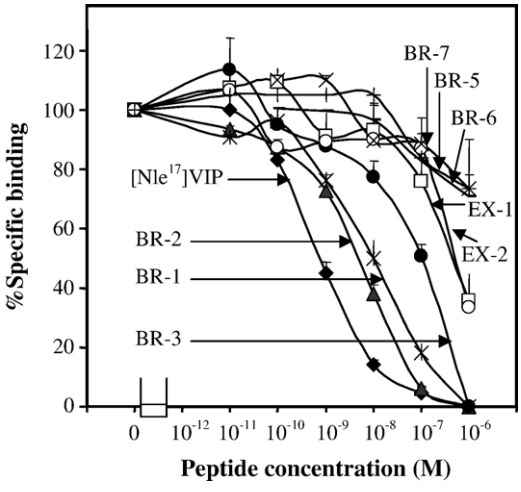

Fig. 1. Dose effects of [Nle^{17}]VIP and some VIP analogs for the inhibition of ^{125}I-VIP binding to HT29 cells. Cells (4×10^5 cells/well) were seeded on collagen-precoated 24 wells and cultured for 2 days. Incubations with radioligand (50 pM, ^{125}I-VIP) were performed at 4 °C during 180 min in the presence of increasing concentrations of unlabelled peptides. Non-specific binding was subtracted for each value. Data are the means (±SEM) of 3–4 independent triplicate experiments.

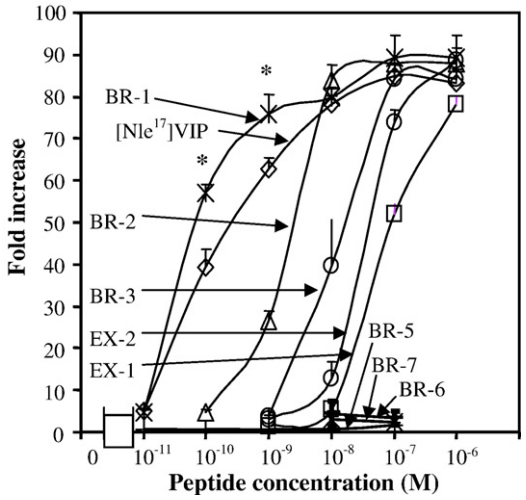

Fig. 2. Dose-dependent effects of [Nle^{17}]VIP and VIP analogs on intracellular cAMP levels in HT 29 cells. HT 29 cells were seeded in 24-well culture plates 3 days before the experiment. Then, intact monolayer cultures were incubated for 30 min. at 37 °C in the presence of the indicated concentrations of [Nle^{17}]VIP and VIP analogs in DMEM containing 0.1 mM IBMX. Intracellular cAMP levels were determined using commercially available ELISA kit. Data are the means of 3–4 independent triplicate experiments. Control cells were treated with saline. The results are presented as the fold increase as compared to control cells. The control cells level of cAMP was 1.5 pmol/10^6 cells. Administration of the 10^{-9} M, 10^{-10} M VIP branched peptide, BR-1 increased cAMP levels significantly (*$P<0.05$ by Mann Whitney U test) higher than [Nle^{17}]VIP.

Peptides were dissolved in double distilled water (DDW) and TFE (trifluroethanol)/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 $[\phi]$ in degrees–cm^2–dmol^{-1}. $[\phi]=[\phi]_{OBS}(MRW/10\ l\ C)$, $[\phi]_{OBS}$ 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.

2.6. Data analysis

Results are presented as the means±SEM of 3–4 different triplicate experiments. Mann Whitney non-parametric U test was used to evaluate the cAMP assay results. The criterion for statistical significance was two tail probability of less than 5% (*$P<0.05$).

3. Results

3.1. Peptide synthesis

All synthesized peptides were obtained in high yields (80–85%). Peptide purity (assessed by RP-HPLC) was higher than 97%. Mass spectra data of the HPLC-purified peptides is given in Table 1 and was in agreement with the expected mass. Amino-acid ratios were very close to the expected values and are presented in Table 2. All analogs were synthesized to include Nle^{17} replacing Met^{17} as this replacement has been shown either to increase [23] or not change [28] the peptide binding/activity while enhancing its stability against oxidation.

3.2. Binding experiments

HT-29 cells, expressing only the VPAC1 receptor, were chosen for the analysis of binding of the various analogs [29]. Data obtained from binding experiments allowed the determination of the IC_{50}, the concentration of unlabelled peptide leading to half maximal inhibition of the ^{125}I-VIP specific binding. [Nle^{17}]VIP potently inhibited specific ^{125}I-VIP binding to HT29 cells (Table 3, Fig. 1). The IC_{50} obtained for [Nle^{17}]VIP ($IC_{50}=7\times10^{-10}$ M, Table 3) is in good agreement with that reported by Lelievre for VIP on HT-29 cells, namely, $IC_{50}=6\times10^{-10}$ M [27]. Native VIP inhibited the specific binding of ^{125}I-VIP with an equal IC_{50} to that of [Nle^{17}]VIP (data not shown). The two extended VIP analogs

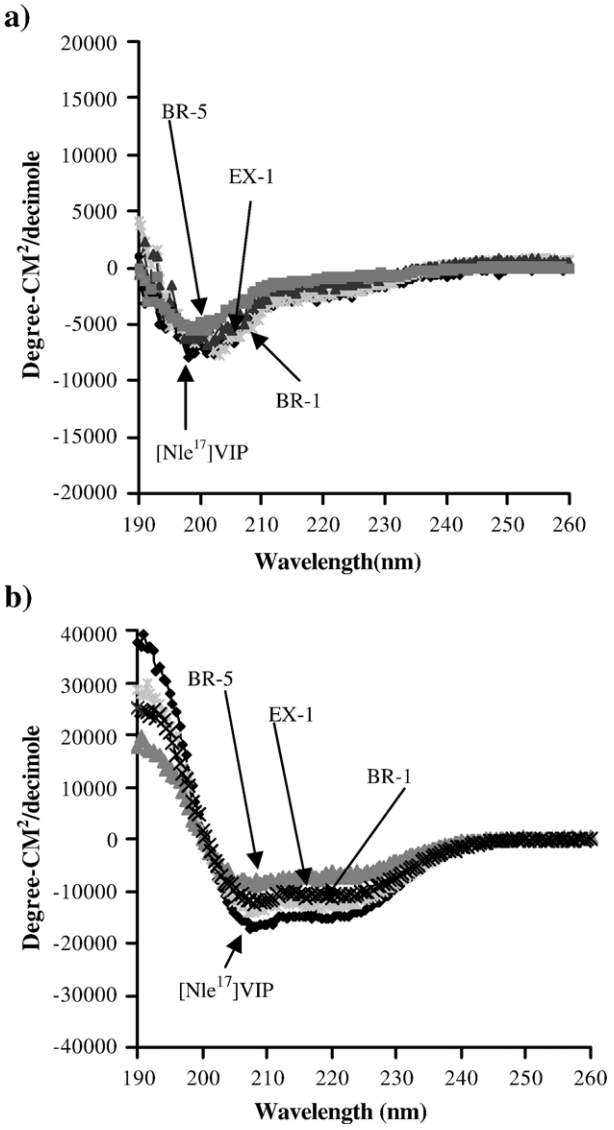

Fig. 3. Circular dichorism spectra of [Nle^{17}]VIP and three VIP analogs with multiple N-terminal (BR-1, BR-5 and EX-1) in DDW(a) and in 40% TFE (b). Similar spectra were revealed by all VIP analogs with multiple N-terminal. Elipticity is reported as the mean residue elipticity $[\phi]$ in degrees–cm^2–dmol^{-1}. $[\phi]=[\phi]_{OBS}(MRW/10\ l\ C)$. $[\phi]_{OBS}$ 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.

demonstrated similar IC_{50} values (Fig. 1, Table 3), higher than 10^{-7} M. The affinity of the extended VIP analogs towards the VIP receptor was approximately 700-fold lower as compared to [Nle^{17}]VIP (Table 3). In contrast, several branched VIP analogs (i.e., BR-2, BR-1, BR-3) demonstrated markedly lower IC_{50} as compared to the extended VIP analogs (Fig. 1, Table 3). Peptide BR-2 had the highest affinity towards the VIP receptor among the branched peptides, only 6-fold lower than [Nle^{17}]VIP. Peptide BR-1 had an IC_{50} value slightly higher (2-fold) than that of peptide BR-2 (Table 3). Peptide BR-3 had an IC_{50} value which was 1 magnitude higher than BR-1 ($IC_{50}=1\times10^{-7}$ M, 1×10^{-8} M, respectively). Despite their high resemblance to the aforementioned peptides, the branched peptides BR-6 and BR-7 did not inhibit even 50% of the ^{125}I-VIP specific receptor binding at a concentration of 1 μM (Fig. 1, Table 3). The peptide BR-5 had the identical C-terminal (VIP_{9-28}, Table 1) and N-terminal sequences (VIP_{1-7}) as that of the branched peptide BR-2. However, whereas the N-terminal domain (VIP_{1-7}) was included two times in the peptide BR-2 through the use of one Lysine, it was included four times in peptide BR-5 through the use of three Lysine moieties. In contrast to the peptide BR-2, which demonstrated the highest affinity towards the VIP receptor among all the VIP analogs tested, peptide BR-5 reduced ^{125}I-VIP specific receptor binding only by 20% at a concentration of 1 μM (Fig. 1, Table 3).

3.3. Effects of [Nle^{17}]VIP and VIP analogs on intracellular cAMP levels

The effects of [Nle^{17}]VIP and VIP analogs on cAMP intracellular levels were assayed following 30 min incubation with the peptides in dose response experiments (Fig. 2, Table 3). [Nle^{17}]VIP was highly efficient in elevating cAMP basal levels (~80-fold increase as compared to the control). The EC_{50} value (concentration required to achieve 50% of the maximal effect) obtained by [Nle^{17}]VIP ($EC_{50}=1\times10^{-10}$ M, Table 3) is in accord with its binding, $IC_{50}=7\times10^{-10}$ M (Table 3). Native VIP was as potent and efficient as [Nle^{17}]VIP in inducing cAMP formation (data not shown). In general, there is a reasonable correlation between the EC_{50} for stimulating enzyme activity and the IC_{50} for inhibiting the ^{125}I-VIP specific receptor binding. Thus, branched VIP analogs that did not reach the IC_{50} at 10^{-6} M (BR-5, BR-6, BR-7) did not increase the cAMP levels (Fig. 2, Table 3). Additionally, several VIP analogs with multiple N-terminal sites in a branched mode (BR-1, BR-2 and BR-3) were clearly more potent than the VIP analogs with multiple N-terminal sites in a linear mode (EX-1 and EX-2) in increasing cAMP levels (Fig. 2, Table 3); this is in agreement with the receptor binding results. The two extended peptides (EX-1 and EX-2) demonstrated low potencies in increasing cAMP levels (their IC_{50} were higher than 10^{-8} M), approximately 350-fold lower as compared to the [Nle^{17}]VIP. The following branched peptides, BR-2 and BR-3, demonstrated reduced potency as compared to [Nle^{17}]VIP (20-fold and 100-fold lower than VIP, respectively, Table 3). This is in accordance with their lower affinity toward the VIP receptor as compared to the [Nle^{17}]VIP (6-fold and 140-fold lower than [Nle^{17}]VIP, respectively, Table 3). The branched peptide BR-1 demonstrated the highest potency among all the VIP analogs examined. Surprisingly, despite the lower receptor affinity demonstrated by this peptide relative to [Nle^{17}]VIP (Fig. 1, Table 3), it showed somewhat superior potency relative to [Nle^{17}]VIP in the cAMP assay (Fig. 2, Table 3). Whereas [Nle^{17}]VIP administration at a concentrations of 10^{-9} and 10^{-10} M resulted in cAMP increase of 63±3 and 39±4-fold, relative to the control, respectively, BR-1 administration yielded significantly ($P<0.05$) higher responses at these concentrations, 76±5, 57±2-fold increase relative to the control, respectively.

3.4. Spectroscopic study by CD

Circular dichorism (CD) analysis was performed in order to elucidate the effect of the N-terminal multiplication on the peptides secondary structure. The CD spectrum of [Nle^{17}]VIP in water revealed one negative minimum at 198 nm (Fig. 3a), indicating on random coil structure. This corroborates with previous studies [30]. The CD curves of the VIP analogs with multiple N-termini in water appeared very similar to that of [Nle^{17}]VIP with one negative minimum around 198 nm (Fig. 3a, data presented for BR-1, BR-5 and EX-1; Similar curves were revealed by all the VIP analogs). The CD spectra of [Nle^{17}]VIP in 40% TFE (Fig. 3b) revealed two negative minima, at 222 nm and at 208 nm, indicating on α helical structure. This is in agreement with previous studies [13,30] that demonstrated that the central part of VIP adopts α helical structure in organic environment. The CD spectrum of the VIP analogs with multiple N-termini in 40% TFE (Fig. 3b) appeared highly similar to that of the [Nle^{17}]VIP, with two minima, at 222 nm and at 208 nm, indicating on α helical structure (Fig. 3b, data presented for BR-1, BR-5 and EX-1; Similar curves were revealed by all VIP analogs).

4. Discussion

This study was designed to evaluate the impact of N-terminal segments multiplication on VIP binding/activity. Nicole et al. [12] demonstrated that His^1 and Val^5, located at the N-terminal of VIP, are directly involved in receptor binding. Here, multiplication of the N-terminal domain was performed by either branching or extending of the VIP peptide. Results showed that branching of the N-terminal of VIP yielded a few analogs that maintained highly potent VIP receptor binding and activation. In contrast, linear extension of VIP led to a sharp decrease in receptor binding and cAMP formation assays. Gourlet et al. [28] suggested that precise positioning of the amino terminal part of the VIP ligand is crucial for the induction of active receptor conformation. Accordingly, we suggest that the reduced binding of the linearly extended peptides to the VIP receptor stems from the N-terminal extension of VIP resulting in poor alignment at the receptor binding site. Furthermore, a similar reduction in receptor binding and activation occurred regardless of the orientation of the extended N-terminal segment (i.e. VIP_{1-6} in EX-1 or VIP_{6-1} in EX-2, Table 1) relative to VIP.

Previously, we showed [23] that [Nle^{17}]VIP was 10-fold more potent as compared to the native VIP in neuroprotection and in cAMP formation assays. In contrast, in the current study [Nle^{17}]

VIP demonstrated equal receptor binding and cAMP activation as compared to the native VIP. This is in agreement with the findings of Gourlet et al. [28]. A possible explanation to the discrepancies relating to the effects of the [Nle^{17}]VIP is that whereas in the current study and in Gourlet et al., study [28] the effects were evaluated on VPAC1 receptor, in our original study [23] effects were examined on astrocytes, which are known to express mainly VPAC2 rather than VPAC1 [31]. However, Nicole et al. [12] demonstrated that the substitution of Met^{17} by Ala^{17} in VIP does not modify binding and cAMP production parameters on the VPAC1 and VPAC2 receptors. Thus, it seems that position 17 does not have a key role in VIP receptor interactions but may be important for VIP stability, as Met can be easily oxidized.

In the past, we prepared a VIP fatty derivative, termed SNV (stearyl-Nle^{17}-VIP), through the addition of a rather large moiety, steric acid (18 carbons), to the N-terminal by an amide bond and the replacement of Met^{17} by Nle^{17} [32]. SNV showed a 100-fold increase in potency as compared to VIP in promoting neuronal survival in a cAMP independent mechanism [23,33]. In these studies SNV activity may have been associated with VPAC2 receptor activation as the system included astrocytes that mainly express VPAC2 and mediate VIP neuroprotection [31]. In contrast, SNV maintained cAMP activation properties on VPAC1 receptor [28]. Furthermore, despite the rather substantial modification of the N-terminal domain of VIP that led to the generation of SNV, the VPAC1 receptor binding and cAMP-induced formation properties demonstrated by this molecule [28] seem to be clearly superior to those achieved by the linearly extended VIP analogs used in the current study. However, since the N-terminal modification in SNV was pronounced lipophilization, the potent receptor binding demonstrated by this compound could have resulted from improved membrane permeability or due to membrane perturbation, as suggested by Granoth et al. [26].

In contrast to the marked decrease in receptor binding and activation in the linearly extended VIP analogs, some branched VIP analogs maintained highly potent VIP receptor binding and activation, rather similarly to that of [Nle^{17}]VIP. This holds true, for example, for the branched VIP analogs, BR-1 and BR-2, which were prepared through Lysine replacement at residues Asn^{9} and Asp^{8}, respectively. Nevertheless, branching following Lysine replacement at residues Phe^{6} and Thr^{7} (BR-7 and BR-6, respectively) yielded VIP analogs that did not reach the receptor IC_{50} even at 10^{-6} M. The negligible receptor binding capacity demonstrated by BR-7 and BR-6 is in accordance with the findings of O'Donnell et al. [16] who performed Ala scan of the VIP derivative (Ro 23-7059) and included Phe^{6} and Thr^{7} in a group of six amino acids that were the most important for the binding activity.

The VIP analogs with multiple N-termini exhibited very similar CD curves in water to the curve of the [Nle^{17}]VIP, displaying a random coil structure. With the addition of 40% TFE, an excellent structure promoting co-solvent, the VIP analogs CD spectra demonstrated high resemblance to that of [Nle^{17}]VIP, indicating on α helical structure. In view of the above, we suggest that multiplication of the VIP N-terminal domain in a branched or in a linear mode does not affect substantially the central α helical part of the VIP molecule. Hence, differences in the receptor binding and activation among the different VIP analogs (branched and extended) in this study seem to result mainly from the altered interaction of the N-terminal of the VIP with the receptor and not from alteration in the central α helical part of VIP. It appears that the branching methodology results in a slight alteration in the alignment of the N-terminal region of VIP at the receptor binding site, not as substantial as obtained with the extending methodology.

Notably, receptor binding/cAMP activation was almost completely lost upon increasing the number of N-terminal branches from two to four (BR-2 vs. BR-5). Thus, it is suggested that the large size of the N-terminal region of the branched analog BR-5 interrupted with the ligand–receptor binding.

The branched peptide BR-1 demonstrated the highest potency in cAMP formation assay among all the VIP analogs examined. Despite the lower receptor affinity demonstrated by this peptide compared to [Nle^{17}]VIP, it showed a slightly superior potency relative to [Nle^{17}]VIP in the cAMP formation assay. Current theories on class 2 G protein-coupled receptor activation suggest that following the scavenging of the cognate peptide by the N-terminal domain of the receptor, there is a subsequent docking of the N-terminal portion of the peptide ligand to the receptor core which is required for triggering receptor activation [11]. The deviation of the peptide BR-1 from the receptor binding–activation correlation might have resulted from the multiplication of a potential epitope, which is responsible for receptor activation in the VIP ligand. Due to its potent cAMP augmenting effect, the branched peptide BR-1 could have potential therapeutic value in several disorders, similarly to VIP [2].

Several studies were performed in order to elucidate the pharmacophore of VIP [12,15,16]. These studies defined which residues in the VIP sequence were critical for receptor binding or for the peptide steric structure, in contrast to those that were not involved in receptor binding or in the peptide steric structure. Literature review reveals a vast number of studies that generated VIP analogs, agonists or antagonists. Most of these analogs were designed on the basis of minor to moderate modifications of the original sequence of VIP, such as replacement of one or more amino acids by chemically similar amino acids [20,34–36] or replacement of L-amino acids by D-amino acids in the VIP sequence. These studies elaborated the understanding of the VIP pharmacophore. We attempted to reveal the impact of larger scale modifications in the N-terminal region of the peptide. We generated two VIP analogs significantly larger (BR-1, BR-2) than the [Nle^{17}]VIP by the addition of 7 or 8 amino acids in the N-terminal part of the peptide. However, these peptides revealed rather comparable binding and activity properties to the [Nle^{17}]VIP. Hence, we suggest that the site in the VIP receptor which interacts with the N-terminal of VIP could be quite tolerant to enlargement modifications in the VIP ligand in this region.

Though docking of VIP in the receptor has not been reported yet, several studies relating to VIP–VPAC1 receptor interactions suggested the following: (1) The central part of VIP with its crucial basic residues may interact with the acidic residues in the electronegative binding groove in the N-terminal domain of the receptor [37]. (2) The C-terminal end of VIP may interact

with the N-terminal domain of the receptor [38,39], possibly with the binding groove identified in this domain. (3) The N-terminal domain of VIP may interact with the core of the receptor that may consist, at least, of the first and second extracellular loops [40]. In view of the above, we suggest that the interaction of the N-terminal domain of VIP with the core of the VPAC1 receptor is relatively flexible and could be tolerant to enlargement modifications. Furthermore, we hypothesize that the branched peptides that carry a very large N-terminal part interact at the surface of the receptor core, probably on extracellular loops of VPAC1 receptor.

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