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SHORT COMMUNICATION

Role of the guanidine group in the N-terminal fragment of PTH(1–11)

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Abstract A series of PTH hybrids containing a diamine $[NH_2(CH_2)_nNH_2; n = 4, 5, 6]$ in the C-terminal position was synthesized based on the H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2 (Har = homoarginine) template. The compounds were pharmacologically characterized at PTH1R receptors for agonist activity.

Keywords SPPS · CD · Peptidomimetics · PTH

Introduction

Recent advances (Hruby 2002) in understanding the chemistry of life have shown that peptide–macromolecular interactions constitute the principal physico-chemical mechanisms by which processes of life are controlled and modulated. Peptide ligands can act as agonists or antagonists at cell-surface receptors and acceptors that modulate cell function and animal behaviour. This area encompasses as much as 50% of current drugs, and is likely to become even more important in the future. The prevalence and diversity of peptides, along with their participation in crucial physiological functions, has renewed the interest in using peptides and peptide derivatives as therapeutic agents. Although peptides have been used extensively to characterize receptor systems, the development of peptides into pharmaceutical products is generally limited due to poor bioavailability. Considerable efforts have been undertaken to improve the pharmacological properties of peptides, mainly by increasing enzymatic stability and improving bioavailability, pharmacokinetics and pharmacodynamics while preserving the structural features required for efficacious and specific activity (Adessi and Soto 2002).

The development of peptide or peptide-mimetic ligands which can target the receptors or the acceptors modulating biological activities is a top priority in biology and medicine. Therefore, establishing systematic structure-based or ligand-based approaches for the design of such ligands has been an important concern (Hruby 2002).

The parathyroid hormone (PTH), an 84-amino acid hormone, plays a vital role in regulating blood calcium homeostasis. PTH-related protein (PTHrP) also plays a critical role in the development of the foetal skeleton (Kronenberg et al. 1997). PTH and PTHrP play their distinct biologic roles yet act through the same G protein-coupled receptor (GPCR), the PTH/PTHrP receptor (PTHR) (Jüppner et al. 1991). As a class B G protein-coupled receptor (Kolakowski 1994) PTHR uses two somewhat autonomous domains to engage its peptide ligands: the amino-terminal extracellular (N) domain, and the transmembrane/extracellular loop or juxtamembrane (J) region. The 1–34 amino acid fragment of PTH has been shown to tightly bind to and effectively activate PTHR. The study of N-terminally truncated PTH(1–34)NH_2 agonist and antagonist analogues has been the subject of extensive research for the development of potent, specific and non-parental bone anabolic drugs (Shimizu et al. 2001a, b; Barazza et al. 2005). The hypothesis of the

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mechanism of the interaction between PTH(1–34) and its receptor involves two principal components: an interaction between the C-terminal domain of PTH(1–34), represented by residues 17–31, and the N-terminal extracellular domain of the receptor and an interaction between the N-terminal, signalling domain of PTH, represented by residues 1–11, and the juxtamembrane (J) region of the receptor, which contains the extracellular loops and seven transmembrane helices (Hoare et al. 2001; Castro et al. 2005; Wittelsberger et al. 2006; Shimizu et al. 2005; Gensure et al. 2005; Dean et al. 2008) .

NMR analyses of PTH(1–34) analogues suggest that the N-terminal portion of PTH, responsible for receptor activation, contains a short α-helical segment from 3 to 13 residues, while a more stable C-terminal α-helical segment is thought to be the principal receptor binding domain. Studies to reduce the peptide size have demonstrated that enhancement of α-helicity in the PTH(1–11) sequence results in potent PTH(1–11)NH_2 analogues (Tsomaia et al. 2004; Lim et al. 2004; Barazza et al. 2005) and that specific modifications can increase signalling potency in peptides as short as 11 amino acids (e.g. S3A, N10Q, L11R or L11hR) (Shimizu et al. 2000).

Therefore, in the context of improving the pharmacological properties of PTH(1–11) analogues, we studied the role of a positive charge at the C-terminal position, which was identified to enhance bioactivity and binding (Shimizu et al. 2000; Lim et al. 2004).

Materials and methods

Materials

All solvents were purchased from commercial sources and used without purification. Reagent grade materials were purchased from GL Biochem (Shangai, China) and Inalco-Novabiochem (Milano, Italy). Molecular masses of final peptides were determined by electrospray ionization mass spectrometry (ESI-MS). Reversed-phase purification was routinely performed on a Shimadzu LC-8A equipped with a Shimadzu SPD-6A UV detector on a Deltapak Waters C_{18}-100 Å silica high performance liquid chromatography (HPLC) column. The operative flow rate was 17 ml/min with a linear gradient of 20–45 (v/v) B over 20 min (A, water + 0.1% TFA; B, 90% acetonitrile + 0.1% TFA). Homogeneity of the products was assessed by analytical reversed-phase HPLC using a Vydac C18 column (218TP510), with a linear gradient 20–45% (v/v) B in 20 min., flow rate 1 ml/min and UV detection at 214 nm. Abbrevations: hR or Har, homoarginine; DCM, dicholoromethane; hLys, homolysine; m[Glu], FmocGluNH$(CH_2)_nNH_2$, $n = 4, 5, 6$.

Synthesis

General Procedure for the synthesis of glutamic acid derivatives (Kruijtzer et al. 1998):

First step The diamine (11 mmol, 5 eq) was dissolved in 100 ml of 10% $NaHCO_3$ in water and acetonitrile (50/50). *Z*-succinimide (2.2 mmol, 1 eq) was added and the mixture was stirred overnight. The reaction was controlled by TLC (first run in CH_2Cl_2/MeOH 9/1 and second run in $CHCl_3$/MeOH/NH_4OH 17% 6/2/2). The solvent was evaporated and the crude material was dissolved in water and filtered. The aqueous solution was extracted with CH_2Cl_2. The organic layers were dried over Na_2SO_4.

Second step The singly protected diamine (0.7 mmol, 1 eq) was dissolved in 20 ml of CH_2Cl_2 and Fmoc-Glu(*Ot*Bu)OPfp (1.0 mmol, 1.5 eq), HOBt (1.0 mmol, 1.5 eq), and DIPEA (2.0 mmol, 3 eq) were added to the solution. The reaction mixture was stirred overnight. The solution was dried, the solid was dissolved in ethyl acetate, and the organic layers were extracted with water, 5% $NaHCO_3$ and brine. The crude material was purified via flash chromatography (ethyl acetate/light petroleum ether 1/1).

FmocGlu(OtBu)NH$(CH_2)_4$NHZ Yields First step, 31%; second step, 83%. ^{1}H-NMR, 400 MHz, $CDCl_3$ (ppm): 7.77–7.3 (13H Fmoc + *Z*); 6.4 (mb, 1H N*H*(amide)); 5.75 (mb, 1H N*H*(Fmoc)); 5.08 (s, 2H CH_2(*Z*)); 4.8 (mb, 1H N*H*(*Z*)); 4.4 (d, 2H CH_2(Fmoc)); 4.22–4.18 (t; mb, 2H *H*-Glu; *H*-(Fmoc)); 3.3–3.1 (2qb, 4H NCH_2); 2.3 (2mb, 2H γCH_2-Glu); 2.0 (2mb, 2H βCH_2-Glu); 1.6–1.4 (m; s, 13H NCH_2CH_2; *t*Bu); calculated mass: 630.31; found mass: 631.12 [M + 1].

FmocGlu(OtBu)NH$(CH_2)_5$NHZ Yields First step, 48%; second step, 66%. ^{1}H-NMR, 400 MHz, $CDCl_3$ (ppm): 7.77–7.3 (13H Fmoc + *Z*); 6.4 (mb, 1H N*H*(amide)); 5.75 (mb, 1H N*H*(Fmoc)); 5.08 (s, 2H CH_2(*Z*)); 4.8 (mb, 1H N*H*(*Z*)); 4.4 (d, 2H CH_2(Fmoc)); 4.22–4.18 (t; mb, 2H *H*-Glu; *H*-(Fmoc)); 3.3–3.1 (2qb, 4H NCH_2); 2.3 (2mb, 2H γCH_2-Glu); 2.0 (2mb, 2H βCH_2-Glu); 1.6–1.4 (m; s, 15H NCH_2CH_2; *t*Bu); calculated mass: 644.31; found mass 645.20 [M + 1].

FmocGlu(OtBu)NH$(CH_2)_6$NHZ Yields First step, 40%; second step, 66%. ^{1}H-NMR, 400 MHz, $CDCl_3$ (ppm): 7.77–7.3 (13H Fmoc + *Z*); 6.4 (mb, 1H N*H*(amide)); 5.75 (mb, 1H N*H*(Fmoc)); 5.08 (s, 2H CH_2(*Z*)); 4.8 (mb, 1H N*H*(*Z*)); 4.4 (d, 2H CH_2(Fmoc)); 4.22–4.18 (t; mb, 2H *H*-Glu; *H*-(Fmoc)); 3.3–3.1 (2qb, 4H NCH_2); 2.3 (2mb, 2H γCH_2-Glu); 2.0 (2mb, 2H βCH_2-Glu); 1.6–1.4 (m; s, 17H NCH_2CH_2; *t*Bu); calculated mass: 658.31; found mass: 659.15 [M + 1].

Third step The cleavage of the *t*Bu protective group on side chain of Glu was carried out in CH_2Cl_2/TFA 10%

(v/v) at room temperature for 2 h. The reaction was checked by TLC (CH_2Cl_2/MeOH 9/1). The mixture was evaporated in vacuum and taken up again with diethyl ether. The purity of the product was checked by ESI-MS.

Solid-phase synthesis

Analogues were prepared by Fmoc solid-phase peptide synthesis on 0.1 mmol of Rink-amide-MBHA resin (Novabiochem, 0.73 mmol/g substitution grade). The side-chain protecting groups were removed under acidic conditions at the time of cleavage of the oligomers from the resin. For all amino acids, the coupling reagents were hydroxy-1H-benzotriazole (HOBt) and N,N,N′,N′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), with diisopropyl ethylamine (DIPEA). For the insertion of [m]Glu, Aib and Val, hydroxy-1H-azabenzotriazole (HOAt) and N,N,N′,N′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HATU), with collidine, were used. The N^{α}-Fmoc protecting group was cleaved using 20% piperidine (v/v) in DMF. The resin-bound peptide was treated with a deprotection and cleavage solution of TFA/TIS/water (95:2.5:2.5 v/v/v) at room temperature for 2 h. After filtration, the filtrate was concentrated under nitrogen and precipitated with methyl *tert*-butyl ether. It was then dissolved in methanol and the *N*-benzyloxycarbonyl group was removed by hydrogenation on Pd/C. After filtration, all crude analogues were purified using reversed-phase C18 HPLC, analytically characterized, and molecular masses were determined by electrospray ionization mass spectroscopy. The yield of the complete synthesis was calculated after purification and lyophilization for all peptides.

Peptide content (pc) The peptide content was measured at 205 nm following the procedure by Tombs et al. (1959). The only amino acids whose side chains contribute moderately to the total peptide absorption at 205 nm are His and Har/Arg.

Biological tests

Adenylyl cyclase assays to test for agonist activity were performed using C20 HEK293 cells stably expressing PTHR seeded at 10^6 cells/well in collagen-coated 24-well plates. Twenty-four hours later, cells were treated with FuGENE 6 Transfection Reagent, CRE-luciferase DNA (100 ng/well), and Renella luciferase DNA (10 ng/well) (acts as internal control for transfection efficiency). Eighteen hours after transfection, the cells were incubated with different concentrations of the compound for 4 h at 37°C, yielding maximal response to luciferase. The luminescent signal was measured using Dual-Glo Luciferase Assay System (Promega) according to manufacturer's protocol. The EC_{50} was calculated by non-linear regression analysis using the GraphPad Prism software (San Diego, CA).

Each peptide concentration was tested in triplicate. Luciferase activity was measured on a Lumat LB 9507 luminometer (E.G&G Berthold). The mean read-out from three wells with identical peptide concentration was used to present the data.

Circular dichroism

Circular dichroism (CD) measurements were carried out on a JASCO J-715 spectropolarimeter interfaced with a PC. The CD spectra were acquired and processed using the J-700 program for Windows. All experiments were carried out at room temperature using HELLMA quartz cells with Suprasil windows and an optical path-length of 0.01 or 0.1 cm. All spectra were recorded using a bandwidth of 2 nm and a time constant of 8 s at a scan speed of 20 nm/min. The signal to noise ratio was improved by accumulating 8 scans. Measurements were carried out in the 190–250 nm wavelength range and the concentration of the peptides was in the 0.07–1.07 mM range. The peptides were analysed in aqueous solution containing 2,2,2-trifluoroethanol (TFE) 20% (v/v). The spectra are reported in terms of mean residue molar ellipticity (degree cm^2 $d-mol^{-1}$). The helical content for each peptide was estimated according to the literature (Yang et al. 1986).

Results

All peptides were synthesized following a general solid-phase peptide synthesis protocol. We used four equivalents of HBTU/HOBt/DIPEA (Konig and Geiger 1970; Carpino 1993) as coupling reagents and double couplings with HATU/HOAt/Collidine (Carpino et al. 1994; Carpino and El-Faham 1994) only for the hindered residues. The series of analogues of PTH(1–11) and modified peptide analogues are listed in the Table 1 with the results of chemical, physical and biological tests.

The synthesis of Fmoc ω-benzyloxycarbonylaminoalkylglutamic acid amides [Fmoc-GluNH$(CH_2)_n$NHZ] was carried out in solution by modifying glutamic acid (Glu) to obtain an orthogonally protected building block that can be anchored to the resin via the γ-carboxyl moiety in solid-phase peptide synthesis (Scheme 1).

This synthetic approach was utilized to generate a series of analogues containing glutamic acid amides of variable chain length to explore the relationship between the distance separating the C-terminal basic function from the peptide backbone and bioactivity. The length of the carbon chain was $n = 4$, 5 and 6, mimicking the side chains of ornithine, lysine and homolysine, respectively.

Table 1 C-terminal modified analogues of PTH(1–11)NH_2

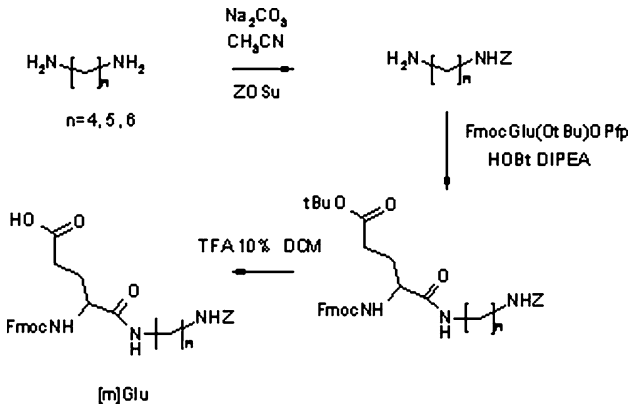
Name	Peptide sequence	MW calc. [M + 1]	MW found [M + 1][a]	R_t (min)[b]	Yield (%)	pc (%)[c]	EC_{50} (nM)[d]
I	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2	1,317.7	1,317.7	12.07	35.4	74	1.0
II	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Lys-NH_2	1,276.7	1,276.4	12.63	10	70	50
III	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-D-Har-NH_2	1,317.7	1,317.7	12.47	38.5	75	75
IV	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln $NH(CH_2)_4NH_2$	1,219.7	1,219.4	13.29	7	66	Not active
V	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln $NH(CH_2)_5NH_2$	1,233.7	1,233.4	13.68	11	79	100
VI	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln $NH(CH_2)_6NH_2$	1,247.7	1,247.4	13.95	12	90	200

[a] Molecular weight is experimental data [M + H^+]

[b] R_t (min) determined with a linear gradient of 20–45 (v/v) B over 20 min (A: water + 0.1% TFA; B: 90% acetonitrile + 0.1% TFA)

[c] pc is the peptide content determined according to Tombs et al. 1959

[d] EC_{50} (nM) is defined as the half maximal effective concentration and is referred to the concentration of peptide which induces a response halfway between the baseline and the maximum. The experimental error on the measurement is ±7.5%



Scheme 1 The solution phase synthesis of glutamic acid derivatives modified at the α-carboxylic function

The α-amino-ω-benzyloxycarbonylaminoalkanes were coupled to the α-COOH of Glu via pentafluorophenyl ester in the presence of HOBt and DIPEA. The modified amino acid was then coupled to the resin and the stepwise elongation of the peptide was carried out on the solid support following published procedures. The coupling of Aib and Val residues was carried out by employing the HOAt/HATU/collidine system (Carpino et al. 1994; Carpino and El-Faham 1994). At the end of the stepwise assembly of the peptide chain, the side-chain protecting groups were removed and the resin-bound peptide was cleaved from the Rink–amide–MBHA resin concomitantly under acidic conditions [95% trifluoroacetic acid (TFA)]. The appreciated advantage of Rink–amide–MBHA resin was to obtain the peptide analogue as C-terminal amide, which transformed the Glu^{10} in the desired residue Gln^{10}. All peptides had the correct molecular weight as determined by ESI-MS mass spectrometry. All crude peptide analogues were purified using reversed-phase C18 HPLC and their structural integrity and purity was assessed by ESI-MS and analytical RP-HPLC, respectively.

Circular dichroism studies were carried out in aqueous solution in the presence of 20% TFE. The presence of a positive band at ~190 nm and negative bands at ~208 and 222 nm indicate a helical conformation for all the modified peptides, a prerequisite for PTH-like activity (Shimizu et al. 2003; Barazza et al. 2005) (Fig. 1).

The ω-aminoalkylamide-containing analogues of PTH(1-11) display only very low adenylyl cyclase activity. The lower activity, with respect to the reference peptide containing Lys^{11}, could be attributed to the absence of the C-terminal amide group or to the absence of chirality of the C-terminal residue.

Discussion

The PTH is composed of 84 amino acids, but its full biological effects are limited to its N-terminal region, i.e. PTH(1–34). To solve the delivery problems and to learn more about how this ligand binds/activates the PTH/PTHrP receptor, more extensive investigations have been undertaken on the structure and function of PTH and PTH/PTHrP, in the last years (Dean et al. 2008). In previous works (Lim et al. 2004; Caporale et al. 2006, 2009), the first six residues of PTH(1–11) analogues were found to be very critical for receptor activation and that an essential feature for bioactivity was an α-helical structure in the N-terminal portion, from residue 3 to residue 10. An α-helix had been suggested as the preferred bioactive conformation of the N-terminal portion of PTH by previous structural studies on PTH(1–34) analogues (Pellegrini et al. 1998; Jin et al. 2000), functional studies on conformationally constrained N-terminal PTH fragments (Gardella and Jüppner 2001), as well as computer-generated models of the PTH–PTHR complex (Rölz et al. 1999; Monticelli et al. 2002). Recently, Lim et al. (2004) described that isolated alanine-rich peptides with charged residues added

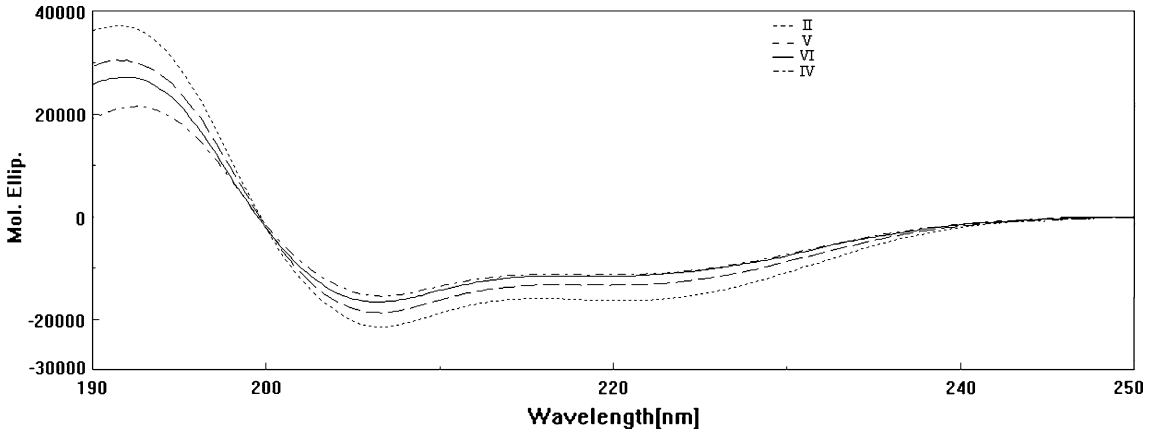


Fig. 1 CD spectra of C-terminal analogues of PTH(1–11)

for solubilization could increase helix propensity and that residues 10 and 11 could play a critical role in the activation both by stabilizing the helix and by improving the binding with the receptor.

The results presented here confirm that the positive charge at the end of the peptide is important for binding. Specifically, the reduced potency of all the analogues studied supports the important role of homoarginine, which replaces leucine of native PTH, in anchoring PTH(1–11) to the receptor, either in the stabilization of the structure (Lim et al. 2004), or in receptor binding (Shimizu et al. 2001a). Molecular modelling suggests that this role might involve the insertion of the guanidinium side-chain group between the extracellular ends of TM1 and TM7 (Monticelli et al. 2002).

Compound III, which differs from I only in the chirality of the homoarginine (Har) residue, is 75-fold less active than compound I. This is consistent with the hypothesized crucial role of anchoring the peptide to the receptor. Nevertheless, it is somewhat surprising that the side chain of D-Har should not be able to occupy a similar position to that of the L analogue on the receptor surface, considering that it is a terminal residue with high degrees of conformational freedom. A possible reason for this result might be that rotation around the φ angle of D-Har disrupts a stabilizing intramolecular interaction, such as a hydrogen bond involving its carbonyl oxygen. Alternatively, the anchoring role of Har^{11} is not limited to its side chain, and the correct positioning of the side chain in the D-analogue prevents such interaction.

Based also on this observation, we decided to modify the side chain of the last amino acids to design a simple peptidomimetic, without changing the overall three dimensional structure. As a reference, we synthesized an analogue peptide containing Lys in position 11: [$Aib^{1,3}$, Gln^{10}, Lys^{11}]PTH(1–11)NH_2, to avoid the final guanylation reaction (Table 1). The activity of this analogue was 50-fold lower than that of [$Ala^{1,3}$, Gln^{10}, Har^{11}]PTH(1-11)NH_2 (Shimizu et al. 2000).

A CD study of the synthesized peptides was performed to compare their conformation with the results of the biological tests. CD provides a facile method to characterize possible overall secondary structural changes induced by modifications of residue 11. The conformational properties of the new analogues were investigated in 20% (TFE)/water, as in our previous experiments on potentially bioactive PTH-derived peptides (Barazza et al. 2005; Caporale et al. 2009). The addition of TFE (Goodman and Listowsky 1962) as a co-solvent to aqueous solutions of peptides results in the stabilization of the α-helix conformation. This "TFE effect" has been extensively applied to the study of structure and conformation of model peptides and protein fragments (Buck 1998). Therefore, the observed helicity of these analogues might be confidently correlated with their potential capacity to interact productively with the PTH receptor. The CD results suggest that helicity is required, but not sufficient, to obtain high activity, as the diamine analogue with $n = 4$ was as helical as the analogue with $n = 5$, but, contrary to the latter, it was inactive.

The reduced activity observed for analogues V and VI, containing an amino-pentyl and an amino-hexyl group, respectively, could be justified by a role of the C-terminal amide group. Analogue V, containing the amino-pentyl group ($n = 5$), is the most similar to the analogue containing Lys, differing only for the C-terminal amide group, but its activity is twice as low. The structure of analogue V was highly helical, as required, so the activity loss might be ascribed to a relevant role of the C-terminal amide in receptor interaction or in helix stabilization.

Our results suggest that the distance between the backbone and the positive charge in the side chain is a second, crucial factor. In fact, the lack of activity of analogue IV, containing an amino-butyl group ($n = 4$), might stem from an unfavourable distance between the positive charge and

the receptor. Also, the 50-fold reduction in activity displayed by analogue II relative to analogue I might reflect the presence of a shorter side chain.

Both the length of the positively charged side chain and its correct orientation seem to be necessary for productive receptor interaction. The distance of the positive charge from the peptide backbone should be higher than four methylene groups. Five methylenes are optimal when the charge is linked to the main chain, while the same distance is too short if placed in the side chain, as demonstrated by the loss of potency on going from the Har^{11} to the Lys^{11} analogue. Specifically, the 100- and 200-fold reduction in potency observed for analogues V and VI, containing amino-pentyl ($n = 5$) and amino-hexyl groups ($n = 6$) in position 11, compared to the analogue containing homoarginine, suggests that the search for potent PTH analogues should consider the modification of the L-Har side chain, to keep the positive charge in the correct orientation.

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