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Structure–function relationship studies of PTH(1–11) analogues containing D-amino acids

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ABSTRACT

Parathyroid hormone (PTH) is an 84-amino acid peptide hormone. Produced in the parathyroid glands, it acts primarily on bone and kidney to maintain extracellular calcium levels within normal limits. It has been shown that the 1–34 amino acid fragment of PTH is sufficient to bind and activate the PTH type-I receptor. Recent investigations focusing on the interaction of N-terminal fragments of PTH with PTH type-I receptor showed that certain modifications can increase signalling potency in peptides as short as 11 amino acids. To understand the role of the side chains of all the amino acid residues in PTH(1–11), we synthesized all-D PTH, three retro-inverso analogues of the most active modified PTH(1–11), H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2, and we substituted every L-AA of the latter with the corresponding D-AA, obtaining a library of PTH(1–11) analogues that were tested as agonists. The library was synthesized by SPPS, employing the Fmoc protocol. The biological tests showed that the activity of the D-Har11 analogue is of the same order of magnitude of that of the most active modified PTH(1–11). This behaviour is paralleled by an increase of the helical content on going from the D-Val^2 to the D-Har^{11} analogue. This is in agreement with previous work where a correlation between activity and helical content has been demonstrated. The importance of a positively charged group in the C-terminal position is shown to be independent of the configuration of the C^{α}-carbon.

1. Introduction

In mammals, parathyroid hormone (PTH) (Kronenberg et al., 1997), an 84-amino acid hormone, plays a vital role in regulating the concentrations of ionized calcium and phosphate in blood and extracellular fluids. PTH-related protein (PTHrP) plays a critical role in the development of the fetal skeleton (Chorev and Rosenblatt, 1994). The biological actions of both these peptides are mediated by the PTH/PTHrP receptor (or PTH1 receptor) (Jüppner et al., 1991), a family B G protein-coupled receptor (Kronenberg et al., 1997) expressed on the surface of bone and kidney target cells. It has been shown that the first 34-amino acid fragment of PTH is sufficient for in vivo bioactivity, and to reproduce biological responses characteristic of the native intact PTH (Kronenberg et al., 1997). Clinical studies have demonstrated that PTH(1–34) is a powerful bone anabolic agent able to restore bone mineral density.

The prevailing view of the biologically relevant conformation of PTH(1–34) includes a flexible molecule with no tertiary structure (Gardella and Jüppner, 2001; Shimizu et al., 2002). NMR analyses of PTH(1–34) analogues in a variety of polar and non polar solvents suggest that the N-terminal portion of PTH, known to be responsible for receptor activation, contains a short α-helical segment from Ser^3 to Lys^{13}. In addition, there is a more stable C-terminal α-helical segment (from Arg^{20} to Val^{31}), where the principal receptor binding domain is located. These two helices are separated by two hinge-like motifs located around positions 12 and 19 (Scian et al., 2006). 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 (Potts and Gardella, 2008; Tsomaia et al., 2004; Barazza et al., 2005). Based on mutagenesis studies and on the position and shape of the binding sites for residues in position 2, 5 and 8, high helicity has been suggested to be essential for receptor activation (Monticelli et al., 2002; Shimizu et al., 2000a,b). Furthermore, location of residue 8 on the same face of the helix as Ile^5, as well as the position of Val^2 projecting toward the third extracellular loop (EC3) have been hypothesized to be fundamental requirements for the activation of PTH1 receptor (Shimizu et al., 2000a,b).

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Introduction of conformational constraints, such as α-amino isobutyric acid (Aib), into peptides can improve their activity and receptor binding selectivity (Hirschmann, 1991; Gante, 1994; Kessler et al., 1995).

To probe the effect of an enantiomeric topological disposition of the critical side chains in PTH, we synthesized first of all an analog of PTH(1–34) containing all D-amino acids. To investigate the importance of the relative position of specific side chains, we focused on the most active PTH(1–11) analogue, H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2 (Shimizu et al., 2001a,b). Initially, we introduced retro-inverso modifications and consequently, we substituted every L-amino acid with the corresponding D-amino acid, obtaining a library of PTH(1–11) analogues that were tested as agonists (Table 1).

Replacement of each residue by its optical isomer provides useful information regarding possible turn positions as only certain turn types can accommodate either L or D-residues, and D-amino acids may confer resistance to proteolytic degradation. Another important advantage for peptide drug development is that analogues containing D-amino acids have been found to be significantly less immunogenic than those containing their L-amino acid counterparts (Gill et al., 1963; Borek et al., 1965; Quintana et al., 2007).

The replacement of Met with Nle is known to be well tolerated with no loss of binding affinity (Rosenblatt et al., 1976) and avoids methionine oxidation, which would result in a decrease of the biological response (Frelinger and Zull, 1984). The replacement of Leu^{11} with Arg^{11} enhanced autoactivation (Shimizu et al., 2000b) in the PTH1 receptor/[Arg^{11}]PTH(1–11) tethered system, which lacks most of the extracellular N-terminal domain (N-ECD) of PTH1 receptor, thus leading to the hypothesis that neither the 1–181 N-ECD of the receptor nor the C-terminal portion of PTH(1–34) are essential for bioactivity. Moreover, the presence of Arg^{11}/Har^{11} turned some analogues of PTH(1–11) into potential agonists (Shimizu et al., 2001a,b), while PTH analogues truncated at position 10 displayed poor binding and low activity (Shimizu et al., 2004).

2. Materials and methods

2.1. Synthesis

A general synthetic protocol for the solid-phase synthesis of allDPTH(1–34) and peptidic analogues of PTH(1–11)NH_2 was used with an automated peptide synthesizer (model 348 Ω, Advanced ChemTech, Louisville, KY). The analogues were prepared using the Fmoc methodology with a Rink Amide MHBA Resin (Novabiochem) (0.73 mmol/g loading) as a solid support (Carpino et al., 1994) and using Fmoc (*N*-(9-fluorenyl)methyloxycarbonyl) main chain protecting group chemistry. All amino acids and reagents were commercially available and were used without previous purification. Only D-Homoarginine was synthesised according to the literature (Caporale et al., in press). The first amino acid was anchored to the resin using the HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)/HOBt (1-hydroxybenzotriazole)/DIPEA (diisopropylethyl amine) protocol (Knorr et al., 1989; Carpino, 1993). Deprotection of Fmoc from the α-amino groups was achieved under standard conditions, with a 20% piperidine solution in DMF. The following amino acids were coupled in the same way as the first one. The coupling of Aib and Val was accomplished with the more potent condensation reagent HATU (2-(1H-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (Carpino et al., 1994, 1995). To improve the incorporation yield of hindered amino acids, a longer reaction time and double couplings were used.

Table 1
Har = Homoarginine; Aib = α-amino isobutirric acid; Abu = 4-amino butirric acid; Amc = aminocapronic acid (or 6-amino hexanoic acid); Nle = norleucine.

Name	Peptide sequence
AllDPTH (1–34)	H-D-Ser-D-Val-D-Ser-D-Glu-D-Ile-D-Gln-D-Leu-D-Nle-D-His-D-Asn-D-Leu-Gly-D-Lys-D-His-D-Leu-D-Asn-D-Ser-D-Nle-D-Glu-D-Arg-D-Val-D-Glu-D-Trp-D-Leu-D-Arg-D-Lys-D-Lys-D-Leu-D-Gln-D-Asp-D-Val-D-His-D-Asn-D-Phe-NH_2
RI	H-D-Har-D-Gln-D-His-D-Nle-D-Leu-D-Gln-D-Ile-D-Glu-Aib-D-Val-Aib-NH_2
RII	H-Abu-D-Gln-D-His-D-Nle-D-Leu-D-Gln-D-Ile-D-Glu-Aib-D-Val-Aib-NH_2
RIII	H-Amc-D-Gln-D-His-D-Nle-D-Leu-D-Gln-D-Ile-D-Glu-Aib-D-Val-Aib-NH_2
RP	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2
D2	H-Aib-D-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2
D4	H-Aib-Val-Aib-D-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2
D5	H-Aib-Val-Aib-Glu-D-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2
DAllo5	H-Aib-Val-Aib-Glu-*allo*-d-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2
D6	H-Aib-Val-Aib-Glu-Ile-D-Gln-Leu-Nle-His-Gln-Har-NH_2
D7	H-Aib-Val-Aib-Glu-Ile-Gln-D-Leu-Nle-His-Gln-Har-NH_2
D8	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-D-Nle-His-Gln-Har-NH_2
D9	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-D-His-Gln-Har-NH_2
D10	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-D-Gln-Har-NH_2
D11	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-d-Har-NH_2

2.1.1. Peptide deprotection, cleavage from the resin and purification

The resin-bound peptides were treated with a deprotection and cleavage solution of TFA (trifluoroacetic acid)/TIS (triisopropylsilane)/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. Peptide purification was performed by reverse phase HPLC on a Deltapak Waters C_{18}-100 Å silica column (solvent A: water + 0.1% TFA; solvent B: Acetonitrile 90% + 0.1% TFA) with a linear gradient of 10–35% (v/v) solvent B over 15 min. Peptide homogeneity (>95%) was determined by analytical HPLC on a Vydac C_{18} (218TP510) column using the same solvents with a linear gradient of 10–90% (v/v) solvent B over 30 min. Molecular masses were determined on a Perseptive Biosystems MARINER™ API-TOF spectrometer.

2.2. 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 optical path-lengths of 0.01 cm and 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 performed in the wavelength range 190–250 nm and the concentration of the peptides was in the range 0.07–1.07 mM. The peptides were analyzed in aqueous solution containing 20% (v/v) 2,2,2-trifluoroethanol (TFE). The spectra are reported in terms of mean residue molar ellipticity (deg cm^2 $dmol^{-1}$). The helical content for each peptide was estimated according to the literature (Yang et al., 1986).

2.3. NMR measurements

NMR spectra were recorded at 298 K on a BRUKER AVANCE DMX-600 spectrometer. The experiments were carried out in H_2O/TFE-d_3 $(4:1)_{v/v}$. The sample concentration was approximately 1 mM in 600 µl of solution. The water signal was suppressed by pre-saturation during the relaxation delay. The spin systems of all amino acid residues were identified using standard DQF-COSY (Rance et al., 1983) and CLEAN-TOCSY (Bax and Davis, 1985a; Griesinger et al., 1988) spectra. In the latter case, the spin-lock pulse sequence was 70 ms long. The sequence-specific assignment was accomplished using the rotating-frame Overhauser effect spectroscopy (ROESY) experiment (Bax and Davis, 1985b), using a mixing time of 150 ms. In all experiments, the spectra were acquired by collecting 400–512 experiments, each one consisting of 32–256 scans and 4 K data points.

Spectral processing was carried out using the program XWINNMR. Spectra were calibrated against the TMS (tetramethylsilane) signal.

2.4. Activity assays

Human Embryonic Kidney (HEK 293) cells stably transfected with recombinant PTH1-receptor (HEK293/C20 cell line) were used (Pines et al., 1994). The PTH1 receptor is a class II GPCR, which couples strongly to the adenylyl cyclase (AC)–protein kinase A (PKA) signalling pathway. In HEK 293 cells, the cAMP response element (CRE) of Luciferase was transfected using CRE-Luc plasmid. This response element (CRE), which is a recognition site of certain transcription factors, interacts with CREB (CRE-binding protein), which is regulated by cAMP. Thus, the activity of PTH1 receptor is monitored by using CRE positioned upstream of the luciferase gene. Activation of the receptor causes an increase in intracellular cAMP, which is able to activate protein kinase A to phosphorylate CREB. The luciferase concentration within cells is increased when phosphorylated CREB is bound to the CRE consensus sequence, causing an increase in transcription rate of luciferase gene (Fan and Wood, 2007).

2.4.1. Cell culture and CRE-Luc transfection

HEK293/C20 cell line were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2. The cells were subcultured by treatment with Versene every week and the medium was changed every 3–4 days. 24 h before transfection, the cells were seeded at 10^5 cells/well in 24-well, collagene coated plates. On the following day, the cells were treated with FuGENE 6 Transfection Ragent (1 µl/well), CRE-Luc plasmid (0.2 µg/well) in 0.5 ml/well Opti-Mem 1, serum free medium, according with the manufacturer's recommended procedure.

DMEM, fetal bovine serum, Opti-Mem I, and PBS were from Life Technologies, Inc.; FuGENE 6 Transfection Reagent was purchased from Roche Diagnostic (Indianapolis, IN); Passive Lysis Buffer, 5× from Promega Corporation (Madison, WI); Biocoat Collagen I 24-well plates from Becton Dickinson (Bedford, MA) while the other tissue culture disposable and plasticware were obtained from Corning (Corning, NY). D-Luciferin, potassium salt was obtained from Molecular Probes (Eugene, OR).

2.4.2. Luciferase assay

About 18 h after CRE-Luc plasmid transfection, the cells were rinsed with PBS (phosphate buffer saline) and the transfection medium was replaced by 225 µl/well of Dulbecco's modified Eagle's medium. 25 µl/well of peptides solutions at different concentrations (from 10^{-7} to 10^{-3} M to obtain final concentrations between 10^{-8} and 10^{-4} M) in PBS supplemented with 0.1% bovine serum albumine were then added to the wells and incubated at 37 °C for 4.5 h, yielding maximal response to luciferase. After this time, the medium was aspirated and the cells lysed by gentle shaking with 200 µl/well of Passive Lysis Buffer. The cells were transferred to labelled low binding Eppendorf tubes, centrifuged for 2 min and 80 µl/tube of supernatant were transferred to individual sample glass tubes. Luciferase activity was measured using a Lumat LB 9507 luminometer (EG&G Berthold). This instrument automatically injects defined volumes of two solutions, A and B, with compositions described below. Initially, a Solution 0 is prepared, containing 25 mM glycylglycine, 15 mM $MgSO_4$ and 4 mM ethyleneglycol-bis(β-amminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) in deionized water. Solution A is 0.2 mM D-luciferin in Solution 0. Solution B is 0.02 M K_3PO_4, 2.5 mM ATP and 1 mM dithiothreitol in Solution 0. The instrument adds 100 µl of Solution A and 300 µl of Solution B to a sample tube, and performs the measurement for 20 s. All the CRE-Luc experiments were carried out in triplicates.

2.4.3. Data calculation

Calculations and data analysis were performed using Microsoft Excel 2000 and GraphPad Prism, Version 3.0.

3. Results

The synthesized library of analogues of PTH(1–11)NH_2 is summarised in Table 2.

An allDPTH(1–34) analogue was synthesized following general protocol for the solid-phase peptide synthesis. We used HBTU/DIPEA coupling reagents in 4 equivalents and we used double couplings only for the hindered residues. In the synthesis of retro-inverso analogues, the reversed direction of the peptide introduced a steric difficulty in the coupling of the first amino acid to the solid support. In fact, Aib is a hindered, hydrophobic amino acid and can promote aggregation on growing peptide chains, which may complicate the synthesis. Moreover, the synthetic scheme was complicated by the second and third residues, which were Val and Aib, respectively (Goodman and Chorev, 1979). These problems were overcome through the use of HATU, the more efficient coupling reagent for SPPS introduced by Carpino (1993). We observed that a double coupling of Aib with HATU yielded a good resin loading in the presence of DIPEA as a base. However, during the course of a third coupling partial deprotection of the first Aib occurred, maybe because of the basic character of DIPEA (Carpino and El-Faham, 1994; Carpino et al., 1994, 1995). This event was observed by MS analysis where the signal of Fmoc-Aib-Aib-NH_2 was detectable. After the synthesis of the first fragment, Fmoc-Aib-Val-Aib-Resin, the more common peptide coupling system, HBTU/HOBt, was used and the coupling of the following amino acids proceeded with good yields, using four equivalents of coupling reagent agents against one equivalent of resin. From the HPLC analysis of the crude peptide, it was possible to detect good yields and an efficient synthesis of the retro-inverso peptides.

The synthesis protocol adopted for the D-Scan was Fmoc methodology. D-Homoarginine was prepared by direct guanylation of lysine. Typically, the synthesis of guanidines involves the reaction of amines with an electrophilic amidine species. We rapidly synthesized fully protected homoarginine (Har). The *N*-α-Fmoc-lysine was subjected to guanylation with bis Boc-triflyl guanidine in DCM, prepared according to a reported method (Drake et al., 1994; Feichtinger et al., 1998), in the presence of trimethylsilyl chloride (TMS-Cl) as a silylating agent to afford *N*-α-Fmoc-bisBoc urethane protected homoarginine (Caporale et al., in press). The crude peptides were purified by HPLC.

Even if the allDPTH(1–34) did not show any biological activity, CD analysis displayed exactly the same structure of all-L PTH(1–34), but with a left-handed α-helix (data not shown).

The conformational proprieties of retro-inverso analogues were investigated by CD in 20% TFE/water, as in our previous experiments on PTH-derived peptides (Barazza et al., 2005). The addition of TFE

Table 2
Overall yields, analytical retention times, and calculated and experimental molecular weights of the synthesized peptides.

Name	Yield (%)	t_R (min)	MW Calc $[M+H^+]$	MW Exp $[M+H^+]$
allDPTH(1–34)	62	19.95	4080.74	4080.20
RI	23.7	17.82	1317.73	1317.72
RII	46.9	18.00	1232.66	1232.33
RIII	50.4	18.10	1260.71	1260.22
RP	33.5	16.98	1317.73	1317.72
D2	50.5	16.90	1317.73	1317.72
D4	61.2	17.05	1317.73	1317.72
D5	35.4	16.96	1317.73	1317.72
DAllo5	42.0	16.96	1317.73	1317.72
D6	34.5	16.67	1317.73	1317.72
D7	35.8	17.50	1317.73	1317.72
D8	39.0	17.70	1317.73	1317.72
D9	44.8	16.70	1317.73	1317.72
D10	34.4	17.32	1317.73	1317.72
D11	38.5	17.40	1317.73	1317.72

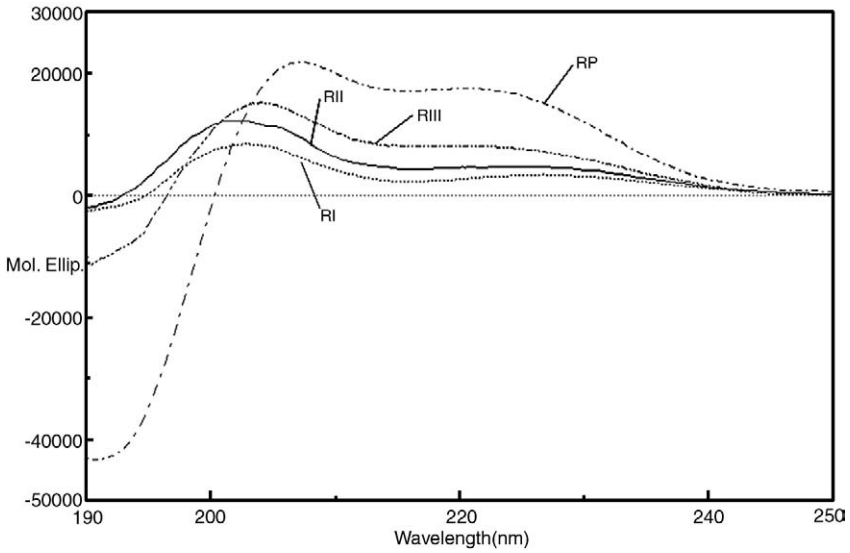

Fig. 1. CD spectra of retro-inverso PTH(1–11) analogues. PTH(1–11) is taken as a reference. Its CD spectrum is inverted in sign to better compare it with the retro-inverso PTH(1–11) analogues series.

(Goodman and Listowsky, 1962) as a co-solvent to aqueous solutions of peptides results in stabilisation of the α-helical conformation. This "TFE effect" has been extensively applied to the conformational study of model peptides and protein fragments (Buck, 1998). The CD spectra of the retro-inverso analogues are reported in Fig. 1. For this series of analogues, the CD spectrum of peptide H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2 (taken as reference) was inverted in sign to better compare it with the retro-inverso PTH(1–11) analogues series (Fig. 1).

Retro-inverso peptides exhibited an ordered structure, but with a low percentage of α-helix structure, which is a requirement to activate the receptor. The lack of α-helical structure may be ascribed to the absence of C^{α}-tetra-substituted amino acids in the N-terminal position. Aib and its analogues are well known to induce and stabilize 3_{10}/α-helices (Crisma et al., 2002).

The CD spectra (Fig. 2) show a decreased helix content for all analogues relative to the reference peptide. Analogues D4, D5, D-*allo*-5, D7, D8 and D9 showed the most marked reduction. The CD spectra of D10, D6 and D2 reflect a more ordered structure. Substitution of L-Har^{11} with D-Har^{11} in the D11 analogue has almost no effect on the α-helical conformation. NMR measurements were performed on all analogues. Fig. 3 shows the chemical shift differences of the C^{α} protons with respect to the values of the random coil conformation (Pastore and Saudek, 1990). The negative difference values ($\Delta\delta < -0.1$ ppm) commonly identify a helical segment.

In drug discovery, it is important to determine if and to what extent candidate compounds inhibit or induce any activity. Luciferase activity is measured in the presence of ATP (the required luciferase substrate) so that light output varies with ATP concentration (Cali et al., 2008). As a general approach, light intensity is correlated to the chemical concentrations of components of luciferase pathway reactions. When the experiment is designed properly, the light intensity can be used to associate an observable parameter with a molecular process. Biological assays were carried out on all peptides after a preliminary control of structure by CD analysis and the results are reported in Table 3.

For the all-D-PTH(1–34), there was no detectable biological activity, even if the CD spectra showed an ordered structure. It was just observed that exhibited an ordered structure. The biological tests on retro-inverso analogues showed low activity, in line with the low percentage of α-helical structure detected. In Fig. 4, the low activity curves of the retro-inverso analogues in the luciferase assay are compared to that of the reference peptide.

The biological assays carried out on the D-scan analogues showed that the activity of the D11 analogue is of the same order of magnitude

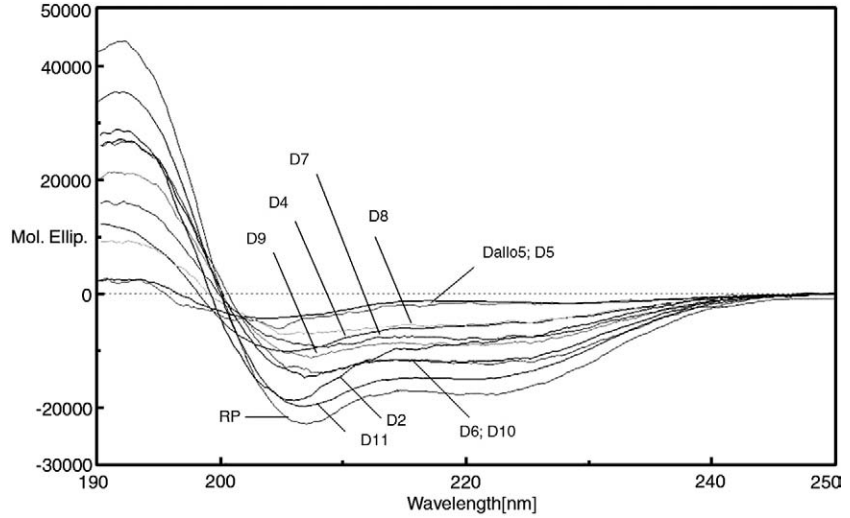

Fig. 2. CD spectra for all analogues in the D-scan series. RP is the reference peptide: H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2.

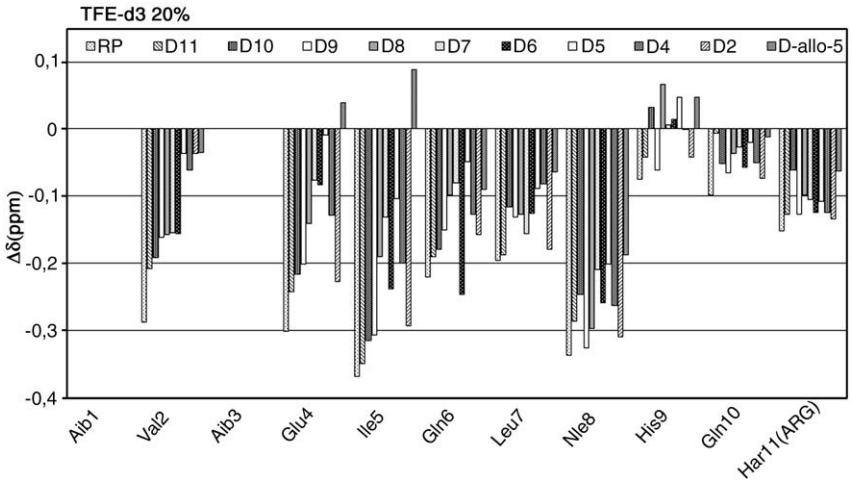

Fig. 3. Secondary chemical shifts of D-scan analogues. HarPTH is the reference peptide, all-L-PTH(1–11) analogue.

as the most active modified PTH(1–11) analogue, taken as RP (Fig. 4). The other analogues have low activity, but they do not lose the capacity to interact with the PTH1 receptor.

This behaviour is in line with our conformational analysis by secondary chemical shift studies and CD spectra, where we observed an important α-helical structure only for D11.

4. Discussion

Our first approach to address the pharmacological properties of the N-terminal PTH fragment was the use of retro-inverso modifications (Schumacher et al., 1996). In this method, the L-amino acids of a peptide are replaced with D-amino acids, and the direction of the peptide chain is reversed. The topology of the side chains is largely maintained and such structures are resistant to proteases. The retro-inverso modification does not introduce conformational restrictions relative to the native peptide. The small number of retro-inverso derivatives that actually show activity comparable to that of the original compounds indicates that the peptide backbone also plays an important role for recognition by the receptor or that there are indeed differences in the side-chain topology (Cushman et al., 1990) in the two classes of compounds. Three retro-inverso analogues, which differ in the N-terminal residue, and also the allDPTH(1–34) analogue were prepared. None of these peptides exhibited biological activity (Fig. 4). In the case of the allDPTH(1–34) analogue, this result is the obvious consequence of the enantiomeric disposition of all the side chains, which does not affect the amount of the secondary structure, but significantly alters the interaction with the receptor. The loss of activity of the retro-inverso analogues can be ascribed to a lack of the α-helical structure (Fig. 2), caused by the absence of C^{α}-tetra-substituted amino acids in the N-terminal position. In fact, Aib and its analogues are well known to induce and stabilise 3_{10}- and α-helices (Martin et al., 2001). The small residual activity of RI points to the importance of the guanido group for receptor interaction.

Our second approach entailed a D-scan of fragment PTH(1–11) to investigate the effect of the single substitution of every residue with the corresponding D-amino acids. The CD spectra (Fig. 2) show a low helix content for analogues D4, D5, D-*allo*-5, D7, D8 and D9, and this result was confirmed by NMR analysis. This effect is maximized for D-*allo*-Ile^5 and $DIle^5$, possibly because these are the only β-branched amino acid in the sequence, and therefore, the most hindered amino acids. Interestingly, the exchange of the β-stereo-center in D-*allo*-Ile^5 altered the structure dramatically and induced a random coil, as seen both by CD and α-proton secondary chemical shifts NMR. Nevertheless, both changes at residue 5 resulted in similar, low reduction of biological activity with respect to the other analogues.

Introduction of a D-residue in peptides D2, D6, D10, and D11 indicate a smaller effect on the structure, and the results of biological tests showed a reduction in activity, rather modest for D11 and more marked for the other analogues, especially D6. From the CD spectra of these analogues, the presence of some contribution of 3_{10}-helix or β-turn cannot be excluded. The relatively high helix content in peptide D2 can be ascribed to the influence of the Aib residues flanking D-Val^2. Specifically, Aib^3 is able to induce a helical conformation that starts after D-Val^2, as indicated by the fact that this residue does not adopt a helical conformation (Fig. 3). The C-terminus is not very ordered, in any of the peptides considered. Correspondingly, substitution with a D-residue in peptides D10 and D11 does not affects helicity in the C-terminus. The most difficult result to interpret is the high degree of ordered structure found for D6 both by CD and by NMR. In fact, residue 6 seems more helical in the D configuration than in the reference peptide. Possibly, the exchange in topology between the α proton and the side chain could favour an H-bond between the side chain of Gln^6 and that of one of the three C-terminal residues (Tsomaia et al., 2004; Shimizu et al., 2003).

In conclusion, these studies confirm the importance of the topological arrangement of residues (1–9) of PTH(1–34) for biological activity (Shimizu et al., 2001a,b). The contribution of the guanidine group in the C-terminal position was shown to be independent of the configuration of C^{α}-carbon. The 1000-fold reduction in affinity and potency previously observed upon removal of Har^{11} highlights the importance of this residue (which replaces Leu of hPTH) in receptor

Table 3
The experimental error on the measurement is ±7.5%.

Name	EC_{50}(μM)[a]
AllDPTH	Not active
RI	Not active
RII	Not active
RIII	Not active
RP	0.001
D2	7.0
D4	2.0
D5	0.4
DAllo5	0.45
D6	4.0
D7	3.0
D8	2.0
D9	2.0
D10	0.1
D11	0.075

[a] EC50(μM) is defined as half maximal effective concentration and is referred to the concentration of peptide which induces a response halfway between the baseline and maximum.

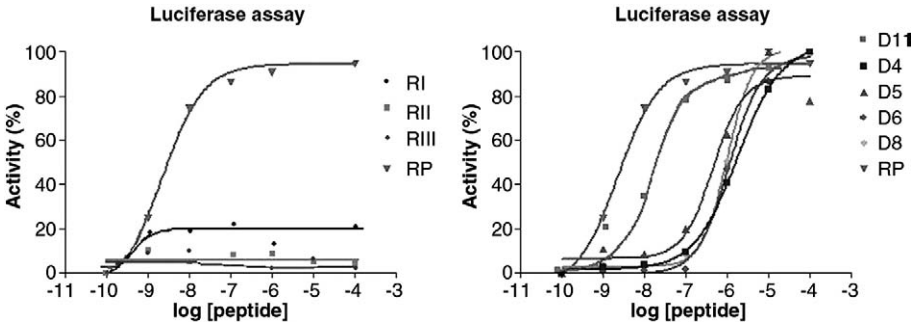

Fig. 4. Full dose–response curves of retro-inverso analogues of PTH(1–11) (left) and of some D-Scan analogues of PTH(1–11) (right). It is possible to observe the low activity of the retro-inverso analogues compared with that of the reference peptide.

binding (Monticelli et al., 2002). In fact, studies on hormone–receptor complex reveal the presence of a H-bond between Arg^{11} of the hormone analog and Glu444 of the receptor (Tsomaia et al., 2004). The formation of this H-bond is not important in PTH(1–34) in which the presence of a higher number of residues is responsible for the interaction with the receptor.

Molecular modeling suggests that the role of Har^{11} might involve the insertion of the guanidinium side-chain group between the extracellular ends of TM1 and TM7 (Shimizu et al., 2001a,b). In PTH (1–11), the stabilization of this position could be critical for the activity of the analogue. Moreover, recent functional and structural (Shimizu et al., 2003) studies on PTH(1–14) analogues suggest that Gln^{10} participates in an intramolecular side chain–side chain interaction with Gln^{6} and thereby stabilizes a local α-helical structure. Loss of this putative stabilizing interaction may thus account for the lack of activity in the PTH(1–9) and other shorter analogues examined thus far.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.03.040.

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