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Analogues of Human Parathyroid Hormone (1–31)NH_2: Further Evaluation of the Effect of Conformational Constraint on Biological Activity

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Abstract—A series of conformationally-restricted analogues of hPTH was prepared, based on the parent peptide agonist, *cyclo*(-Lys[18]-Asp[22])[Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1–31)NH_2 (**2**, $EC_{50} = 0.29$ nM). Truncation of **2** at either the N- or C-termini resulted in peptides with reduced agonist activity as measured by stimulation of adenylate cyclase activity in the rat osteosarcoma cell line (ROS 17/2.8). Alanine- and glycine-scanning at the N-terminus of **2** was consistent with data previously obtained on linear hPTH(1–34). Other locations within the primary sequence of hPTH(1–31)NH_2 were evaluated by the placement of the [*i*, *i*+4] lactam constraining element. Ring size and lactam orientations at the 18–22 positions were also examined.

Human parathyroid hormone (hPTH), an 84 amino acid peptide, is the major regulator of calcium levels in the body and exerts its biological action by interacting with the G-protein-coupled PTH/PTHrP receptors (PTHRs) located on cells in the GI tract, in the kidney, and in bone.[1] It is now well-recognized that most of the known biological activities of hPTH can be accommodated by shorter N-terminal peptides, including hPTH(1–34) and hPTH(1–31)NH_2.[2] Recently, it has been shown that hPTH(1–34) can serve as a potent osteogenic agent in animal models as well as in human subjects and has therefore attracted considerable attention as a possible anabolic agent for the treatment of post-menopausal or Type I osteoporosis.[3] Because of its increasing appeal as a therapeutic agent, investigations have been focused on unraveling the molecular basis for the activity of hPTH. Specifically, researchers have sought to understand the conformation by which the N-terminus of hPTH interacts with its endogenous seven-helical transmembrane (7-TM) receptor.[4]

The ability to obtain specific structural data on the interaction of medium-length peptide hormones with their endogenous 7-TMs is complicated by factors such as the flexibility of the peptide and the loss of structural integrity of the receptor when isolated from its membrane environment. We,[5] and others,[6–8] have chosen to approach this problem by preparing a series of conformationally-restricted analogues of hPTH(1–34)NH_2 and hPTH(1–31)NH_2, the shortest known fragment of hPTH with recognized anabolic activity in the ovariectomized rat model (OVX) of osteoporosis.[9]

We previously demonstrated that incorporation of five mutations into the natural sequence of hPTH(1–31)NH_2 (**1**) resulted in a marked increase in in vitro activity as measured by increased cAMP production in the ROS 17/2.8 cell line. Subsequent modification of this peptide by conformationally limiting the midregion of the peptide with the placement of a side chain-to-side chain

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lactam bridge between the non-natural Lys^{18} and Asp^{22} residues provided a further improvement in activity. *Cyclo*(Lys^{18}-Asp^{22})[Ala^1,Nle^8,Lys^{18},Asp^{22},Leu^{27}] hPTH (1–31)NH_2 (**2**, Fig. 1), when administered by daily subcutaneous injection, was shown to be an osteogenic agent in the OVX rat model of osteoporosis.[10] This discovery led us to synthesize several other novel analogues of **1**, culminating in the most conformationally-constrained analogue reported to date, *tricyclo*(Lys^{13}-Asp^{17},Lys^{18}-Asp^{22},Lys^{26}-Asp^{30})[Ala^1,Nle^8,$Asp^{17,22}$,Lys^{18},Leu^{27}]hPTH(1–31)NH_2 (not shown) which contains three [*i*, *i*+4] side chain-to-side chain lactam bridges spanning residues 13–30.[5] The ability of this analogue to stimulate and bind to the PTH receptor, as well as results from CD studies have led to the hypothesis that the N-terminus of hPTH interacts with its cognate receptor in an "extended" or linear motif as opposed to the "U-shaped" or paired-helix conformation which has been interpreted from NMR spectroscopy under a variety of solvent conditions. In this communication, we report further results obtained in our investigation towards discovering a low molecular weight agonist of the PTH/PTHrP receptor.[11]

C- and N-Terminal Truncations of Peptide 2

At the outset of this investigation, we hypothesized that the C-terminal amphiphilic helix of hPTH(1–34), comprising residues 20–34, served to nucleate a helical conformation at the N-terminal activation domain of the peptide. It has been suggested that Leu^{24}, Leu^{28}, and Val^{31} are critical to high affinity receptor binding in linear PTH species due to their ability to stabilize the hydrophobic face of the C-terminal amphiphilic helix.[12] Whitfield and co-workers have shown that substitution of the hydrophilic Lys^{27} residue of hPTH(1–34) with a hydrophobic Leu^{27} residue, the naturally-occurring residue in PTHrP, afforded more potent PTHR agonists by reinforcing the amphiphilic character of the helical receptor binding domain.[13] We proposed that stabilization of this helical structure by inclusion of the 18–22 lactam-bridge might allow for C-terminal truncation past Leu^{28} without significant loss of receptor activation. For comparative purposes, we also performed a series of C-terminal truncations on the parent, linear peptide hPTH(1–31)NH_2 (**1**, Table 1).[14] Removal of Val^{31} from peptide **2** resulted in a 2-fold reduction in activity in the ROS cell-based assay (cf., **7**). The 29- and 28-mers of **2** (**8** and **9**, respectively) were an order of magnitude more potent than their non-bridged counterparts (**4** and **5**, respectively) while truncation past residue Leu^{28} afforded an analogue (**10**) with less then 200 times the activity of peptide **2**, but significantly more active than hPTH(1–27)NH_2 (**6**).

We next turned our attention towards truncation at the N-terminus of peptide **2**. As described in Table 2, removal of the first N-terminal residue (cf., **11**) resulted in a significant loss of the peptide's ability to stimulate AC in the ROS 17/2.8 cell line. The (3–31) analogue (**12**) is nearly equipotent with **11**, however truncation past residue 3 afforded peptides (cf., **13** and **14**) with no detectable levels of AC-stimulating activity in this assay. These *N*-truncation results are fully consistent with those obtained for analogues of linear PTH(1–34) confirming that the first two N-terminal residues are mandatory for strong agonist activity in linear as well as in these conformationally-restricted peptides.[15] In fact, deletion of up to six terminal residues from the N-terminal activation domain of the linear peptides is a proven strategy for the preparation of PTH/PTHrP receptor antagonists.[16]

Alanine and Glycine Scans of Peptide 2

We performed both alanine and glycine scans in the N-terminal region of the lactam-containing peptide (**2**) to evaluate how the *cyclo*(Lys^{18}-Asp^{22}) bridge might effect the agonist activity within a series of structurally-similar peptides as well as to compare these results to those

Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-NH_2 **(1)**

Ala-Val-Ser-Glu-Ile-Gln-Leu-**Nle**-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-**Lys**-Glu-Arg-Val-**Asp**-Trp-Leu-Arg-Lys-**Leu**-Leu-Gln-Asp-Val-NH_2 **(2)**

Figure 1. Primary sequence comparison of hPTH(1–31)NH_2 (**1**) and peptide **2**. (Bolded residues indicate point mutations; the bracket indicates the position of the lactam bridge.).

Table 1. C-Terminally truncated analogues of hPTH(1–31)NH_2 (**1**) and peptide **2**

Peptide	AC activity[a] (EC_{50}, nM)
HPTH(1-31)NH_2 (**1**)	4.7
HPTH(1-30)NH_2 (**3**)	47
HPTH(1-29)NH_2 (**4**)	60
HPTH(1-28)NH_2 (**5**)	95
HPTH(1-27)NH_2 (**6**)	>10,000
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Cyclo(Lys^{18}-Asp^{22})[Ala^1,Nle^8,Lys^{18},Asp^{22},Leu^{27}]hPTH(1-31)NH_2 (**2**)	0.29
Cyclo(Lys^{18}-Asp^{22})[Ala^1,Nle^8,Lys^{18},Asp^{22},Leu^{27}]hPTH(1-30)NH_2 (**7**)	0.45
Cyclo(Lys^{18}-Asp^{22})[Ala^1,Nle^8,Lys^{18},Asp^{22},Leu^{27}]hPTH(1-29)NH_2 (**8**)	2.10
Cyclo(Lys^{18}-Asp^{22})[Ala^1,Nle^8,Lys^{18},Asp^{22},Leu^{27}]hPTH(1-28)NH_2 (**9**)	2.38
Cyclo(Lys^{18}-Asp^{22})[Ala^1,Nle^8,Lys^{18},Asp^{22},Leu^{27}]hPTH(1-27)NH_2 (**10**)	63.8

[a]Adenylyl cyclase stimulation in ROS 17/2.8 cells (mean results from duplicate assays).

Table 2. N-Terminally truncated analogues of peptide **2**

Peptide	AC Activity[a] (EC_{50}, nM)
Cyclo(Lys[18]-Asp[22])[Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1-31)NH_2 (**2**)	0.29
Cyclo(Lys[18]-Asp[22])[Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(2-31)NH_2 (**11**)	163
Cyclo(Lys[18]-Asp[22])[Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(3-31)NH_2 (**12**)	108
Cyclo(Lys[18]-Asp[22])[Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(4-31)NH_2 (**13**)	>10,000
Cyclo(Lys[18]-Asp[22])[Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(5-31)NH_2 (**14**)	>10,000

[a]Adenylyl cyclase stimulation in ROS 17/2.8 cells (mean results from duplicate assays).

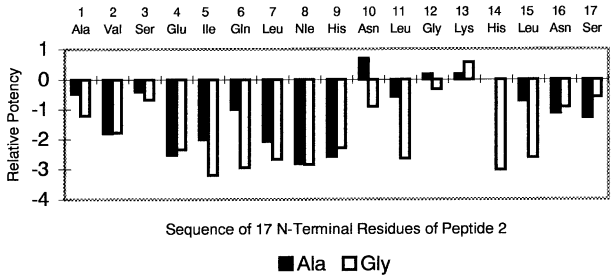

Figure 2. Ability of [Ala^n] and [Gly^n] analogues of peptide **2** to stimulate adenylyl cyclase in ROS 17/2.8 cells. Plot is relative potency, defined as $-\log[EC_{50}(\textbf{Analogue})/EC_{50}(\textbf{2})]$, versus amino acid residue position. Data represent mean cAMP values of duplicate samples.

reported on linear hPTH(1–36)NH_2.[17] Alanine is recognized as a strongly helix-stabilizing residue which has a high level of occurrence within helical regions of natural proteins while glycine is only weakly helix-forming and is poorly represented in protein helical structures due to its flexible nature.[18,19]

As described in Figure 2, neither alanine nor glycine substitution within the first 17 amino acid residues resulted in a substantially more potent agonist than the lactam-containing parent (**2**). These results correlate well with those obtained by researchers at Sandoz for the alanine scan of hPTH(1–36) amide demonstrating that, like the linear hormone, alanine substitution is poorly tolerated from position 3 to 9 while the mid-region of the peptide (residues 10–17) is much more tolerant towards alanine substitution.[17] Alanine substitution at positions 10, 12, 13, and 14 of peptide **2** had no detrimental effect on the agonist activity of the peptide, suggesting that this region of the peptide is either not involved in direct ligand–receptor interactions, or that this region is adopting a specific conformation which is not disturbed by alanine substitution.

Like the alanine scan results, glycine substitution also appears to be poorly tolerated within the 3–9 region of the peptide. However, different results were obtained within the central 10–17 region. Glycine substitution at positions 10, 11, 14, and 15 resulted in decreased AC activity. Substitution of a glycine residue at position 13 formed a Gly[12]Gly[13]-containing peptide of comparable potency to peptide **2.** This result is intriguing since the presence of this glycine dyad would be expected to destabilize a helical structure within a linear peptide. Positions 12 and 13 have been shown to be tolerant towards a range of hydrophobic substitutions within the context of both PTHR agonists and antagonists.[20] Mierke et al. have suggested that Gly[12] may be a hinge residue located between two helical stretches of the hormone's secondary structure.[7] We,[5] and others,[21] have demonstrated that incorporation of a Lys[13]-Asp[17] side chain-to-side chain lactam bridge into the sequences of hPTH and hPTHrP can result in analogues with improved in vitro performance suggesting that this region of the peptide may be helical in its relevant bioactive conformation. Gly[12] is a conserved residue in all the known PTH and PTHrP species and recent results reported by Jin et al.[22] suggest that a helical conformation around Gly[12] is essential for full biological activity.

Other Lactam-Restricted Analogues of hPTH(1–31)NH_2 (1)

Our initial selection of the Lys-Asp lactam bridge in peptide **2** was based on reports suggesting that the ring size and orientation of this constraint was optimal for the stabilization of an α-helix,[23] the secondary structural element thought to be essential for both receptor binding and activation. Undoubtedly, the correct choice of the lactam-based constraint of medium-length peptides will be specific to the parent peptide (i.e., for helix stabilization)[24] and/or the peptide–receptor interaction (i.e., improved receptor binding and/or activation).[25] We therefore prepared a number of side chain-to-side chain lactams spanning the 18–22 positions of hPTH(1–31)NH_2 to evaluate the effect on PTHR activation in the ROS 17/2.8 cell line.

Reversal of the lactam orientation afforded an analogue (**15**) with only moderately reduced activity ($EC_{50} = 0.77$ nM) compared to **2** (Table 3).[26] The *cyclo*(Orn[18]-Glu[22]) analogue (**16**) which maintains the same 20-membered ring size of the Lys-to-Asp-bridged peptides (**2** and **15**) was nearly 6 times less potent than **2**. Both 19-membered Orn-Asp lactams (**17** and **18**) were significantly less active than **2**; a 19-membered ring is calculated to be the minimal ring size which will accommodate the α-helical conformation.[27] Finally, relaxation of the conformational constraint by linking the Lys[18] residue with the naturally-occurring Glu[22] residue provided the most active analogue discovered in this investigation (**19**, $EC_{50} = 0.097$ nM). Taken together these results suggest that the orientation of the amide bond as well as the size of the macrocycle may have significant effects either on the overall peptide conformation or on specific ligand-receptor interactions at this region of the peptide.

Next we prepared a set of hPTH(1–31)NH_2 analogues by varying the location of the side chain-to-side chain lactam constraint.[8,28] Barbier et al. have demonstrated that the putative salt bridges formed between residues Glu^{22}-Lys^{26} and Lys^{26}-Asp^{30} can be replaced by lactam bridges thus providing potent agonists of the PTHR.[6] Similarly, we have determined that the 18–22 lactam constraint is also well-tolerated within agonist peptides (cf., **2**, Table 1). Further lactam scanning along this face of the helical peptide provides the *cyclo*(Lys^{14}-Asp^{18})-containing peptide (**20**, $EC_{50} = 0.19$ nM) and the *cyclo*(Lys^{10}-Asp^{14})-containing peptide (**21**, $EC_{50} = 21.8$ nM, Table 4). The N-terminal activation domain of PTH and PTHrP is postulated to extend to residue 10[29] therefore the reduced activity of the (Lys^{10}-Asp^{14})-lactam-containing peptide (**21**) may be related to interference of the bridge with specific ligand–receptor interactions. Photoaffinity cross-labeling and subsequent molecular modeling of the ligand–receptor complex have provided evidence that this region of the peptide is positioned close to the extracellular loop regions of the receptor.[30] The tolerance of the receptor to the 14–18 bridge (cf., **20**) suggests not only that these residues may not interact directly with the receptor but that this region of the peptide may prefer to adopt a helical conformation when bound to the PTHR.

Chorev et al. have demonstrated that a *cyclo*(Lys^{13}-Asp^{17})-bridge is well-tolerated within the context of PTHrP(1–34)-derived agonists and antagonists.[21] We have extended our investigation to include other possible [*i*, *i* + 4] lactams positioned along this second face of the C-terminal helix (Table 4). The (Lys^{17}-Asp^{21})-, (Lys^{21}-Asp^{25})-, and (Lys^{25}-Asp^{29})-containing peptides (**22**, **23**, and **24**, respectively) were well tolerated but were all weaker agonists compared to the 18–22 lactam-containing peptide (**2**).

Since we completed this study, Jin et al.[22] have reported the crystal structure of hPTH(1–34) which presents the peptide as an extended α-helix with a slight bend in the mid-section. Although the peptide is fully helical, it is best represented as a twisted belt comprised of two amphiphilic helices extending from residues 6–20 and residues 21–33, respectively. From this structure, it was surmised that the mid-region lactam bridges observed to increase biological activity are located on either the convex (13–17 lactam) or concave (cf., **2** and **20**) faces of the extended helix. The lactams which reduce activity (cf., **21**, **22**, **23**, and **24**) lie on the sides of the helical arc causing either disruption of the helical structure or interference with ligand–receptor interactions.

The use of conformationally-restricted analogues of peptide hormones can be a valuable tool to gain insight into the minimum structural requirements for bioactivity. We have demonstrated that a helix-stabilizing [*i*, *i* + 4] lactam bridge spanning positions 18-to-22 of hPTH is functionally-active on PTHR-expressing ROS 17/2.8 cells. Removal of residues at the N-terminus of **2** afforded analogues with greatly diminished agonist potential in a fashion similar to what has been reported for the linear hPTH(1–34)NH_2 series. Truncation at the C-terminus of **2** provided analogues that were substantially more active than their linear counterparts; however, activity dropped off markedly following removal of Leu^{28} thus reinforcing the hypothesis that this residue is directly involved in ligand–receptor interactions.

Alanine substitution at the N-terminus of **2** revealed a close correlation to data obtained on linear hPTH(1–36)NH_2 suggesting that the mid-region lactam did not negatively impact the bioactive conformation of the peptide. The intolerance of the PTHR to alanine substitution within the 3–9 region of **2** suggests the loss of discrete ligand–receptor interactions as opposed to loss of helical structure, since Ala is frequently helix-stabilizing. Glycine substitutions were less tolerated at the N-terminus of peptide **2** except at position 13.

This investigation has also demonstrated the tolerance of the PTHR towards conformational constraint in the C-terminal region of hPTH(1–31)NH_2. Specifically, placement of lactam bridges on the face of the

Table 3. Biological activities of hPTH(1-31)NH_2 analogues **15**–**19**

Peptide	Ring size	AC activity[a] (EC_{50}, nM)
cyclo(Asp^{18}-Lys^{22})[Ala^1,Nle^8,Asp^{18},Lys^{22},Leu^{27}]hPTH(1-31)NH_2 (**15**)	20	0.77
cyclo(Orn^{18}-Glu^{22})[Ala^1,Nle^8,Orn^{18},Leu^{27}]hPTH(1-31)NH_2 (**16**)	20	1.63
cyclo(Orn^{18}-Asp^{22})[Ala^1,Nle^8,Orn^{18},Asp^{22},Leu^{27}]hPTH(1-31)NH_2 (**17**)	19	16.73
cyclo(Asp^{18}-Orn^{22})[Ala^1,Nle^8,Asp^{18},Orn^{22},Leu^{27}]hPTH(1-31)NH_2 (**18**)	19	17.08
cyclo(Lys^{18}-Glu^{22})[Ala^1,Nle^8,Lys^{18},Leu^{27}]hPTH(1-31)NH_2 (**19**)	21	0.097

[a]Adenylyl cyclase stimulation in ROS 17/2.8 cells (mean results from duplicate assays).

Table 4. Biological activities of lactam-containing hPTH(1-31)NH_2 analogues **20**–**24**

Peptide	AC activity[a] (EC_{50}, nM)
cyclo(Lys^{14}-Asp^{18})[Ala^1,Nle^8,Lys^{14},Asp^{18},Leu^{27}]hPTH(1-31)NH_2 (**20**)	0.19
cyclo(Lys^{10}-Asp^{14})[Ala^1,$Nle^{8,18}$,Lys^{10},Asp^{14},Leu^{27}]hPTH(1-31)NH_2 (**21**)	21.8
cyclo(Lys^{17}-Asp^{21})[Ala^1,$Nle^{8,18}$,Lys^{17},Asp^{21},Leu^{27}]hPTH(1-31)NH_2 (**22**)	4.25
cyclo(Lys^{21}-Asp^{25})[Ala^1,$Nle^{8,18}$,Lys^{21},Asp^{25},Leu^{27}]hPTH(1-31)NH_2 (**23**)	3.85
cyclo(Lys^{25}-Asp^{29})[Ala^1,$Nle^{8,18}$,Lys^{25},Leu^{27},Asp^{29}]hPTH(1-31)NH_2 (**24**)	4.78

[a]Adenylyl cyclase stimulation in ROS 17/2.8 cells (mean results from duplicate assays).

amphiphilic helix containing residues 14, 18, 22, 26, and 30 affords analogues with improved in vitro activity presumably by stabilizing the helical secondary structure in the receptor binding domain of the hormone. Placement of the lactam bridges along a second face of the C-terminal helix defined by residues 17, 21, 25, and 29 afforded less active species possibly due to unfavorable interactions between the lactam bridge and the receptor. This investigation together with those from other conformationally-restricted hPTH analogues can provide useful information for the development of other potent agonists of the PTHR for treatment of postmenopausal osteoporosis and other disorders of cell calcium regulation.

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