Published in final edited form as:

J Med Chem. 2017 November 09; 60(21): 8816–8833. doi:10.1021/acs.jmedchem.7b00876.

Development of Potent, Protease-Resistant Agonists of the Parathyroid Hormone Receptor with Broad β Residue Distribution

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Abstract

The parathyroid hormone receptor-1 (PTHr1) is a member of the B-family of GPCRs; these receptors are activated by long polypeptide hormones and constitute targets of drug development efforts. Parathyroid hormone (PTH; 84 residues) and PTH-related protein (PTHrP; 141 residues) are natural agonists of PTHr1, and an N-terminal fragment of PTH, PTH(1–34), is used clinically to treat osteoporosis. Conventional peptides in the 20–40-mer length range are rapidly degraded by proteases, which may limit their biomedical utility. We have used the PTHr1-ligand system to explore the impact of broadly distributed replacement of α -amino acid residues with β -amino acid residues on susceptibility to proteolysis and agonist activity. This effort led us to identify new

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NOTES: The authors declare the following competing financial interest(s): S.H.G. is a co-founder of Longevity Biotech, Inc., which is pursuing biomedical applications of α/β -peptides. S.H.G., R.W.C., and T.J.G. are co-inventors on a patent application covering the PTH analogues described here. T.W. is an employee of Chugai Pharmaceutical Co. Ltd. and contributed to this work as an appointee at Massachusetts General Hospital.

Supporting Information Available: Supplementary methods and supplementary figures 1–16 are available free of charge at pubs.acs.org.

PTHR1 agonists that contain $\alpha \rightarrow \beta$ replacements throughout their sequences, manifest potent agonist activity in cellular assays, and display remarkable resistance to proteolysis. The strategy we have employed suggests a path toward identifying protease-resistant agonists of other B-family GPCRs.

INTRODUCTION

Individual cells in multicellular organisms must receive and respond to a wide array of molecular messages from other cells.¹ Much of this information is conveyed by polypeptides, such as growth factors, cytokines or peptide hormones. Aberrant polypeptide-mediated signaling is associated with many human diseases, and therapeutic benefits can be achieved through use of synthetic polypeptides to block excessive signaling (e.g., engineered antibodies or receptor fragments) or to augment insufficient signaling (e.g., peptide hormone analogues).² Although drug molecules based on the poly- α -amino acid backbone frequently display high potency and selectivity, this molecular class manifests intrinsic limitations. Poly- α -peptides that do not adopt a stable tertiary structure, such as peptide hormones and their analogues, tend to be degraded rapidly by proteases.^{3–5}

Concerns about poly- α -peptides as drugs have led to exploration of oligomers with unnatural backbones as a source of molecules that can strongly and specifically engage natural target proteins but resist biodegradation.⁶ This pursuit offers many opportunities for chemical innovation,⁷ but progress to date has been limited, particularly in terms of establishing efficacy *in vivo*^{8–14} and characterizing immune responses.^{15, 16} Our own efforts have focused on peptidic oligomers that contain both α - and β -amino acid residues (α/β -peptides).^{17–21} We recently demonstrated that α/β analogues of two hormones, glucagon-like peptide-1 (GLP-1) and parathyroid hormone (PTH), can serve as potent agonists of the cognate receptors, the GLP-1R and the PTHR1, respectively, in cell-based assays,^{12, 13, 22} and these compounds are active in mice.^{12, 13, 22} The sites of $\alpha \rightarrow \beta$ replacement in these bioactive analogues were limited to the C-terminal portions of PTH(1-34) or GLP-1(7-37), regions that are known to be α -helical in the receptor-bound state.^{23, 24} Related studies have been reported with α/β analogues of vasoactive intestinal peptide, a hormone for which no structural information is available.¹⁴

Although robust strategies have been developed for introducing sets of $\alpha \rightarrow \beta$ replacements that maintain the structure and function of α -helical segments,^{6, 21} there are no guidelines for implementing $\alpha \rightarrow \beta$ replacements in non-helical segments of bioactive polypeptides. This limitation is significant because the receptor-bound conformation is unknown for the N-terminal portion of PTH, and it is possible that this N-terminal segment is partially or entirely non-helical. Moreover, the N-terminal segment of PTH presumably makes intimate contacts within the core of the transmembrane domain of PTHR1 in order to activate the receptor,^{25–27} and such contacts must be maintained after backbone modification if agonist potency is to be retained. Thus, it is not clear that sufficient β residue density can be achieved in the N-terminal segment of a PTH analogue to provide meaningful protection from proteolysis without loss of agonist activity. Introduction of just one or two $\alpha \rightarrow \beta$ replacements in the central portion of a PTHR1 agonist peptide can substantially diminish

receptor activation potency,^{28–30} which shows that proper selection of substitution sites in peptide hormone mimics is crucial.³⁰ In a study of 19-mer peptides that adopt a specific but irregular conformation and bind to vascular endothelial growth factor (VEGF), we found that it was not possible to introduce $\alpha \rightarrow \beta$ replacements without diminution of affinity for VEGF.³¹ Therefore, whether α/β segments can serve as functional mimics of non-helical or conformationally uncharacterized α -peptide segments remains an open question. Here we address this question in the context of the N-terminal segment of PTH(1-34) and related PTHR1 agonist peptides.

This report discloses new α/β -peptide analogues of PTH(1-34), which is the active ingredient in the osteoporosis drug teriparatide.³² Our previous examination¹² of analogues containing only C-terminal $\alpha \rightarrow \beta$ replacements culminated in evaluation of compounds **B5** (renamed **44**, see supporting figure 17 for structure) and **D6** (renamed **21** below) *in vivo*. Both of these α/β -peptides were potent agonists of the PTHR1 as judged by cell-based assays; each matched PTH(1-34) (**1**) in terms of promoting production of the second messenger cyclic adenosine monophosphate (cAMP).¹² Upon subcutaneous injection of **44** or **21**, mice displayed a “calcemic effect” that is characteristic of **1** itself, i.e., both α/β -peptides caused a short-term rise in Ca^{2+} in the bloodstream.¹² This outcome provides an easily and rapidly detected indication that the PTHR1 has been engaged in the animal; calcium response assays can thus be helpful in guiding design of subsequent studies aimed at evaluating potential therapeutic utility over longer-term treatment.³³ PTHR1 agonists are of interest clinically as they can promote bone growth with continued daily administration.^{34, 35} Determining whether new analogues of PTHR1 agonist peptides can induce the acute calcemic response in mice serves as a readily accessible initial test of *in vivo* activity and is well-suited for assessing several candidate compounds in parallel for the prospect of subsequent longer-term evaluation.

Our previous studies revealed that α/β -peptide **21** displayed a calcemic effect of prolonged duration relative to that induced by **1**, but the calcemic effect induced by **44** was more transient than that of **1**.¹² However, both α/β -peptides persisted longer in the mouse bloodstream than did **1**, with **21** persisting longer than **44**. The fact that analogues of **1** containing five or six $\alpha \rightarrow \beta$ replacements retained high agonist activity was significant, given that earlier reports had shown that a single $\alpha \rightarrow \beta$ replacement could substantially disrupt PTHR1 agonist activity.^{28, 29} The lack of correlation between persistence in the bloodstream (**21** > **44** > **1**) and duration of calcemic response (**21** > **1** > **44**) observed for this pair of analogues relative to **1** shows that measuring the lifetime of the analogue in the bloodstream does not provide a complete assessment of that analogue’s *in vivo* pharmacokinetics.

The studies described here were motivated by three related questions. First, is it possible to place β residues near the N-terminus of an analogue of **1** while retaining agonist activity? This question is important because long stretches of α residues, such as the 13 N-terminal residues of **21**, are highly susceptible to proteolysis. Our previous exploration^{11, 12} of analogues of **1** was limited to compounds containing $\alpha \rightarrow \beta$ replacements in the portion known to be α -helical in the receptor-bound state, as demonstrated by the co-crystal structure of PTH(15-34) bound to the extracellular domain of the PTHR1.²³ The design of

these initial analogues of **1** was based on our previous studies of α -helix mimicry with α/β -peptides.²¹ In contrast to the structural information available describing the C-terminal portion of **1** bound to the PTHR1, the receptor-bound conformation of the N-terminal portion of PTH is unknown.³⁵ This segment engages the core of the membrane-embedded heptahelical domain of the PTHR1 to initiate signal transduction.^{36, 37} No high resolution structural information is available for the transmembrane domain of PTHR1; therefore, we lack atomic-level insights regarding interactions between PTHR1 and the N-terminus of PTH. This lack of structural information prevents rational design of PTHR1 agonist peptides containing $\alpha \rightarrow \beta$ replacements near the N-terminus and limits our ability to engage in mechanistic analysis of the results of such replacements. Whether $\alpha \rightarrow \beta$ replacements in the N-terminal region of PTHR1 agonists would be tolerated at all was an open question as we began this work, because previous studies showed that incorporation of non-natural α -residues into the N-terminal portion of PTHR1 agonists frequently causes substantial losses in biological activity.^{38, 39}

Our second motivating question: is it possible to generate potent PTHR1 agonists that contain ring-constrained β residues rather than the flexible β^3 residues employed in α/β -peptides such as **44** and **21**? This question arises because prior α -helix mimicry efforts have shown that β residues with a five-membered ring constraint, such as ACPC or APC (Figure 1), tend to enhance the stability of an α -helix-like secondary structure relative to β^3 residues (i.e., β residues bearing a side chain next to the nitrogen).^{40, 41} In addition, these cyclic β residues enhance resistance to proteolysis relative to β^3 residues.^{42, 43} We expected that cyclic β residues would be tolerated in the C-terminal portion of analogues of **1**,¹³ because this region must adopt an α -helix-like conformation,²³ but it was necessary to test this prediction. Since the receptor-bound conformation of the N-terminal portion of **1** is unknown, the effect on agonist activity of placing cyclic β residues in this region could not be predicted.

The third motivating question: is it possible to identify highly active analogues of **1** that contain β residues throughout the entire sequence? This question is important because of interest in oral delivery of peptide hormone analogues.^{44–46} Patients generally prefer orally delivered drug regimens over parenteral administration, and oral delivery may therefore enhance dosing compliance.^{47, 48} However, poly- α -peptides face two large barriers to oral delivery. First, these molecules must survive the harsh proteolytic environments of the stomach and small intestine, which have been evolutionarily optimized for degradation of poly- α -peptides into amino acids and short peptides that can be absorbed as nutrients.⁴⁹ Second, these molecules must be able to move from the lumen of the intestine into the bloodstream.⁵⁰ Many compounds with MW < 500 and appropriate lipophilicity can move passively from the gut into the blood, but these routes are not generally available to long peptides and proteins.⁴⁵ Previous *in vitro* studies have shown that α/β -peptides containing at least 25% β residues distributed across the sequence can display substantial resistance to cleavage by aggressive proteases.^{17, 19, 40} We therefore wondered whether it would be possible to achieve the necessary β residue density in a potent PTHR1 agonist to slow degradation under conditions encountered in the digestive tract, since such an α/β -peptide

could circumvent the first barrier to oral polypeptide delivery and therefore provide a basis for future efforts to circumvent the second.

RESULTS

$\alpha \rightarrow \beta$ Scan of the N-terminal region of **1**

In order to determine whether the N-terminal portion of **1** can tolerate $\alpha \rightarrow \beta^3$ replacements, we synthesized analogues of this peptide in which each of the first eight residues was individually replaced by the homologous β^3 residue, i.e., by the β^3 residue bearing the same side chain as the original α residue (**2-9**, Figure 1). As with other α/β^3 -peptides described previously, including **44** and **21**, α/β^3 -peptides **2-9** were straightforward to prepare via conventional solid-phase methods; the necessary Fmoc-protected β^3 -amino acid building blocks are commercially available. Peptides **1-9** were evaluated as agonists of the PTHR1 expressed in HEK293 cells (GP2.3 cell line),¹² which have been stably transfected to express this receptor and a modified luciferase that is activated by the second messenger cAMP.⁵¹ Thus, agonist efficacy can be assessed in terms of the degree to which a peptide induces intracellular production of cAMP via luminescence measurements.

The data (Figure 1, Supporting Figure 1) indicate that $\alpha \rightarrow \beta^3$ replacement is well tolerated at position 1 or 2, because analogues **2** and **3** display cAMP-inducing potencies comparable to that of **1**. Substitutions at the remaining six N-terminal positions, however, cause a 10- to 200-fold loss of efficacy (increased EC₅₀ values). The sensitivity of **1** to single-site modifications at residues 3-8 is noteworthy, because we previously observed that incorporation of five or six $\alpha \rightarrow \beta^3$ modifications in the C-terminal portion (as in **44** or **21**) had little or no effect on efficacy as measured by cAMP production.

A second set of analogues (**10-18**) was prepared in which individual N-terminal residues were replaced by a β residue containing a five-membered ring constraint, ACPC or APC (Figure 1). The nonpolar cyclopentane-based ACPC residue⁵² was used at the first eight positions, but APC⁵³ was used to replace His-9. These choices were dictated by the fact that APC bears a basic side chain nitrogen, which might mirror the basic imidazole ring in the His-9 side chain, while none of the preceding eight residues has a basic side chain. We did not prepare the analogue of **1** containing a β^3 substitution at position 9 because Fmoc-protected β^3 -hHis is not commercially available, and the reported synthetic route to this molecule is cumbersome.⁵⁴

Extensive structural precedent has established that β residues with a five-membered ring constraint and *trans* disposition of the amino and carboxyl groups support the adoption of an α -helix-like conformation by α/β -peptides.^{17, 21, 40, 41} ACPC and APC stabilize helical conformations relative to the more flexible β^3 residues. Since the conformation of the N-terminal portion of PTH bound to PTHR1 is unknown, one could not predict whether the cyclic β residues would be tolerated near the N-terminus. It is therefore noteworthy that several of the analogues containing a cyclic β residue matched **1** in terms of potency and efficacy at promoting cAMP production. Particularly striking were the results of ACPC substitution at position 5, 6 or 7, because in each case the resulting analogue was very similar in agonist activity to **1**, while the analogous β^3 substitutions caused a 12- to 160-fold

decline in efficacy. These trends were mirrored in analogous experiments using a cell line (GD5)⁵⁵ that stably expresses a truncated version of the PTHR1 that lacks the extracellular domain (Supporting Figure 2). In these experiments, the introduction of ACPC at positions 1, 5, or 7 resulted in relatively minor losses in biological activity, whereas nearly all other β -substitutions caused moderate to complete losses in bioactivity (Supporting Figure 2). This finding is consistent with previous observations that the biological activities of PTHR1 agonists are more sensitive to N-terminal modifications in assays using cells that express a truncated PTHR1 lacking the extracellular domain in comparison to assays using the full-length PTHR1.^{38, 56}

Based on the results of single cyclic β substitutions, we examined two analogues of **1** with multiple cyclic β residues in the N-terminal region (Figure 2, Table 1, and Supporting Figure 3). Placing ACPC residues at positions 1 and 7 generated an α/β -peptide (**19**) with efficacy indistinguishable from that of the analogues containing only one of the substitutions or from **1** itself. However, introduction of a third ACPC residue, at position 5 (**20**), caused a substantial decline in agonist activity.

α/β -Peptide analogues of **1** containing multiple cyclic β residues and broad β distribution

We prepared α/β -peptide **22** (Figure 2) to determine whether replacement of the six β^3 residues in **21** (previously designated **D6**)¹² with cyclic β residues would affect agonist activity. At four positions we used the hydrophobic cyclic β residue ACPC, and at the remaining two sites we used the basic cyclic β residue APC. It is straightforward to mimic the natural side chain upon $\alpha \rightarrow \beta^3$ replacement, but side chain mimicry becomes more ambiguous when cyclic β residues are employed, particularly since only a few such residues are currently available. Since β^3 -hTrp, β^3 -hMet and β^3 -hPhe all bear hydrophobic side chains, it is logical to replace these β^3 residues with ACPC. APC is a logical replacement for β^3 -hLys because both residues have a basic side chain. The remaining two residues bear acidic side chains. We replaced β^3 -hGlu with APC and β^3 -hAsp with ACPC in an effort to balance concern about creating an α/β -peptide that might be excessively hydrophobic with concern about deviating too far from the natural pattern of charged side chains in **1**. **22** was indistinguishable from **21** as a PTHR1 agonist, based on cAMP production, and both were very similar to **1** itself (Table 1).

We then prepared two α/β -peptide analogues of **1** containing $\alpha \rightarrow \beta$ replacement at nine sites, **25** and **26**, in the hope of identifying a potent PTHR1 agonist with β residues distributed along the entire length (Figure 2). Analogue **25**, which combines the three $\alpha \rightarrow$ cyclic β replacements of **20** with the six $\alpha \rightarrow \beta^3$ replacements of **21**, displayed ~50-fold lower potency relative to the analogue containing only the three N-terminal cyclic β residues (**20**) (Table 1). Since **1** and **21** are very similar in efficacy, the difference between **20** and **25** suggests some sort of anti-cooperativity, in terms of activating the PTHR1, between the sets of $\alpha \rightarrow \beta$ replacements in the N- and C-terminal regions of **25**. Replacing the six β^3 residues of **25** with cyclic β residues, to generate **26**, provided a nearly 10-fold improvement in potency, but **26** nevertheless displays > 200-fold lower potency relative to **1**, as monitored by stimulation of cAMP production (Table 1).

α/β -Peptide analogues of M-PTH containing multiple cyclic β residues and broad β distribution

A new series of α/β -peptides was prepared based on previously reported potent synthetic analogues of PTH, designated M-PTH, that display a modified signaling profile *in vivo* relative to PTH (Figure 2).⁵⁷ M-PTH contains side chain modifications at seven positions relative to PTH (Figure 2). These modifications were identified in the course of developing truncated analogues of PTH that retained high PTHR1 affinity and biological activity.⁵⁸ Two versions of M-PTH have been described, one containing the Ser3→Ala modification and the other with the Ser3→aminoisobutyric acid (Aib) modification. The Ser3→Ala substitution was shown to substantially improve the bioactivity of PTH(1-14) analogues in an early structure-activity relationship study,⁵⁸ while a later study showed that incorporation of Aib at position 3 further enhanced PTHR1 activation potency, at least in PTH(1-14) derivatives.³⁸ Our PTHR1 activation assays showed the Ser3→Ala version of M-PTH(1-34) (**27**) to have slightly reduced cAMP-inducing potency relative to **1**, which is consistent with previous reports on the properties of the Ser3→Aib version of M-PTH(1-34) (Table 1, Supporting Figure 4).⁵⁷ Analogues of **27** bearing only C-terminal $\alpha\rightarrow\beta$ replacements, **30** and **31**, were very similar in efficacy to **27** (Table 1). Thus, the M-PTH scaffold parallels the PTH scaffold in response to C-terminal $\alpha\rightarrow\beta$ replacements (Figure 2, Supporting Figure 4).

The Ser3→Aib version of **27** was used as the basis for analogues containing N-terminal $\alpha\rightarrow\beta$ replacements (Figure 2). In this context, these replacements evoked responses quite different from those caused by analogous replacements in the context of **1**. Thus, α/β -peptides **29**, **34** and **35** were all quite similar to the α -peptide prototype, **27**, in terms of cAMP-inducing potency (Table 1), which contrasts with the substantial declines in potency observed for the corresponding α/β analogues of **1** (Table 2). Particularly striking is the observation that the cAMP potencies of **35** and **27** differ by < 3-fold, while the efficacies of **26** and **1** differ by > 200-fold (Table 2).

We examined the conformational propensities of our analogues of **1** and **27** by circular dichroism (CD), to determine whether such measurements would provide a mechanistic basis for interpreting the impact of structural modifications on biological activity, as was observed in previous studies.^{38, 59} All peptides exhibited CD spectra consistent with significant helicity, as indicated by minima near 222 nm and 208 nm for **1** and analogues containing only a few β residues, and by a single minimum near 208 nm for analogues with higher β residue content (Supporting Figure 5). However, no clear trend was evident between CD intensity, which should correlate with helical propensity, and biological activity. Even the analogue with the weakest bioactivity in this set (**25**) exhibited a CD signature consistent with substantial helix formation for an α/β -peptide.

The agonist activity of **35** represents an affirmative answer to all three of the questions that motivated these studies. This α/β -peptide contains multiple $\alpha\rightarrow\beta$ replacements near the N-terminus, all of the replacements are derived from cyclic β -amino acids, the sites of $\alpha\rightarrow\beta$ replacement are distributed along the entire length, and this analogue approaches **1** in efficacy and potency as an agonist of the PTHR1. Subsequent studies focused on a more extensive characterization of **35** *in vitro* and *in vivo*.

Susceptibility of **35** to proteolysis

1 disappears rapidly from the bloodstream following subcutaneous injection.⁶⁰ This disappearance is due in part to removal via filtration and/or proteolysis by the kidneys.⁶¹ Proteases in homogenized rat kidney preparations rapidly degrade **1**, causing cleavage at multiple sites.⁶² We therefore sought to determine whether the backbone modifications in **35** would enhance stability relative to **1** in rat kidney tissue homogenate.

HPLC analysis was not feasible for these assays because kidney homogenate contains high levels of diverse biomolecules that complicate chromatographic resolution and interpretation. We therefore developed a mass spectrometry-based method for quantifying the concentration of intact **1** or **35** within samples of kidney homogenate as a function of time. This approach indicated that the half-life of **35** is about 15 times longer than that of **1** in rat kidney homogenate (Figure 3a). Sites of hydrolysis in each peptide were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Figure 3b-c). Many degradation fragments were observed for **1** within 5 min of exposure to rat kidney homogenate, and cleavage was so rapid and broadly distributed that few large fragments could be detected after 15 min (Figure 3b). Intact **1** could not be detected by MALDI-TOF MS after the first 5 min. In contrast, only a few fragments of **35** could be detected upon incubation with rat kidney homogenate, and intact α/β -peptide was clearly evident even after 4 hr (Figure 3c).

Mass spectrometric analysis was complemented by the application of a bioassay method for assessing the persistence of agonist activity (Figure 3d). This method measures the capacity of a preparation of peptide exposed to kidney homogenate, or other degradative conditions, to induce cAMP responses in GP2.3 cells as a function of duration of exposure to the degradative agent. This approach was thus used to monitor the loss of peptide bioactivity in kidney homogenate over time (Figure 3d). After 30 min exposure to kidney homogenate, the activity of **1** had declined approximately 1,000-fold, as indicated by a rightward shift in the dose-response plot. In contrast, the activity of **35** in this assay was not significantly altered after 60 min exposure to kidney homogenate. Collectively, these studies revealed that α/β -peptide **35**, containing nine cyclic β residues distributed throughout the backbone, displays substantial resistance to destruction in the presence of rat kidney homogenate relative to α -peptide **1**.

We turned to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) for further evaluation of the proteolytic susceptibility of **35**. The susceptibility of a peptide to degradation by SGF and SIF is not directly relevant for interpretation of the bloodstream persistence of that peptide administered via injection; however, these studies provide insight into the stability of the peptide under conditions that would be encountered following oral administration. Moreover, given the very aggressive proteolytic activity of SGF and SIF, resistance to degradation under these conditions is very likely to correlate with resistance to the milder degradative environment encountered by a peptide in the bloodstream.

The stomach environment promotes very rapid breakdown of proteins into short peptides and amino acids.⁴⁵ SGF, an acidic solution of pepsin, enables one to estimate the lifetime of a peptide or protein in the stomach.⁴⁹ We used SGF to compare **35**, **1**, and **21** in terms of the

likelihood of degradation after oral ingestion. Two assays were employed to assess persistence as a function of time of exposure to SGF. One assay measured the amount of full-length peptide present at various times via HPLC.¹⁷ The second relied on the bioassay described above for kidney homogenate studies to evaluate the capacity of material generated by SGF treatment to activate the PTHR1 as a function of time of exposure to SGF. These assays provide complementary insights. Results from the latter will reflect net activity based on all species present. Some degradation products, e.g., peptides lacking only a few C-terminal residues, may retain PTHR1 agonist activity, while other degradation products, e.g., those lacking only a few N-terminal residues, may display antagonist activity.³⁵

The HPLC assay (Supporting Figure 6) showed that neither **1** nor the α/β -peptide **21** could be detected after 5 sec in SGF. In striking contrast, **35** displayed a half-life of 4 hr in SGF. These observations show that the profoundly destructive nature of the stomach environment toward conventional peptides such as **1** is manifested as well toward α/β -peptides that retain long stretches of α -amino acid residues, such as **21**; however, the degradative actions of the stomach are substantially suppressed toward a peptide with β -residues incorporated along the entire chain, such as **35**. The relative stability of **35** in SGF thus supports our hypothesis that distribution of cyclic β residues along the entire length of an α/β -peptide will confer substantial resistance to proteolysis.

The PTHR1 activation assay results (Figure 4, Supporting Figure 7) further highlight the remarkable resistance of **35** to proteolytic degradation. After 5 sec exposure to SGF, the **1** sample displayed no detectable agonist activity; only very weak PTHR1 agonist activity was observed for the **21** sample after 5 sec in SGF and **24** exhibited a modest loss in activity over 2 min. In contrast, even after 4 hr in SGF the **35** sample displayed very strong agonist activity. Characterization of the peptide fragments produced following incubation in SGF using MALDI-TOF MS (Figure 4e) provides insight into the mechanism by which biological activity is destroyed by SGF. Protease-catalyzed hydrolysis of **21** following residue 7 produces two fragments that, based on previous structure-activity studies,³⁵ are likely to be devoid of biological activity. The higher stability of **25** relative to **21** in SGF probably arises from the presence of the two additional β residues in the N-terminal portion of **25**; the N-terminal portion of **21** is completely devoid of β residues. In addition, the higher stability of **25** is consistent with previous observations that cyclic- β residues can provide superior proteolytic protection relative to β^3 -residues.^{17,40,42}

Simulated intestinal fluid (SIF) contains a mixture of pancreatic enzymes (pancreatin), including trypsin, in pH-neutral buffer.⁴⁹ Even if a peptide survives passage through the stomach, this molecule must persist in the small intestine in order to have an opportunity to move out of the GI tract.^{44, 45} Because of the complex composition of SIF, an HPLC-based assay could not be established. Therefore, only the PTHR1 activation bioassay was employed to assess susceptibility to proteolysis in SIF. **35** and **1** were compared (Figure 4e-f). After 10 min exposure, very little agonist activity was retained in the **1** sample. In contrast, the **35** sample retained considerable activity at this time point.

Activity of **35** *in vivo*

The potent PTHR1 agonist activity observed for **35** in cell-based assays (Figure 2) and the remarkable resistance to proteolysis demonstrated by studies with kidney homogenates, SGF and SIF (Figures 3 and 4) prompted us to ask whether this α/β -peptide would activate the PTHR1 *in vivo*. This question was addressed by monitoring the calcemic effect induced by **35** in mice after subcutaneous injection (Figure 5). This assessment included **22**, an analogue of **1** that represents an intermediate stage in the evolution of **35** from **21**; α/β -peptide **21** itself was previously evaluated *in vivo*.¹²

New α/β -peptides **35** and **22** are indistinguishable from one another and very similar to **1** in terms of the temporal profile of changes in bloodstream Ca^{2+} concentration they induce (Figure 5a). These data indicate that both of the new α/β -peptides are competent to engage the PTHR1 *in vivo*. It is curious, however, that neither **35** nor **22** generates the long-lasting calcemic effect previously observed for **21**²⁰ and for the α -peptide **27**.^{12, 57} As noted above, an excessive calcemic effect is generally regarded as undesirable from a therapeutic perspective in treating osteoporosis.³³

An activity-based bioassay was used to monitor the level of **35** or **22** in the mouse bloodstream after injection (Figure 5b). The conventional method to measure peptide hormone concentration in the bloodstream, involving an enzyme-linked immunosorbent assay, relies on recognition of the peptide by an antibody; however, past work has shown that antibodies that recognize a peptide sequence composed of α -residues are unlikely to cross react with α/β -peptide analogues with the density of β -residues in the analogues studied here.¹⁵ The bioassay we employed is based on the ability of serum samples from mice injected with peptide to activate the PTHR1 in our cell-based assay. It is possible that the α/β PTHR1 agonists bind to albumin in the mouse serum samples, but this binding would be unlikely to alter the biological activity observed for these α/β -peptides relative to that measured for the α/β -peptides in a direct cell-based assay for PTHR1 activation, since the cell-based assays are conducted in a medium that contains substantial bovine serum albumin. Thus, α/β -peptide concentrations deduced in mouse serum samples based on the bioassay are unlikely to be affected by α/β -peptide association with albumin in the serum.

Results from the cell-based assay were converted to serum peptide concentrations using a standard curve (supporting Figure 8). As previously observed,^{12, 57} after injection of **1** (positive control) the level of excess PTHR1-stimulating activity in the mouse bloodstream rises almost immediately and then rapidly subsides, nearly returning to the baseline level after 2 hr. New α/β -peptides **35** and **22** display divergent behavior in terms of bloodstream concentration, in contrast to their similarity in terms of calcemic effect profile (Figure 5a). **22** attains a concentration in the bloodstream that is modestly higher than that of **1**, and **22** seems to persist slightly longer than does **1**. **35**, on the other hand, is barely detectable in the mouse bloodstream at any point after injection. The contrast between the robust but indistinguishable calcemic responses induced by **35** and **22** and the substantial difference between the bloodstream concentration profiles of these two α/β -peptides suggests that bloodstream concentration does not adequately report on the pharmacokinetic behavior of these molecules. This contrast may indicate that **35** rapidly and tightly binds to cell-surface

PTHr1, while the binding of **22** is slower and/or weaker, causing **22** to be detectable in the blood for longer periods than is **35**.

We compared affinities of these α/β -peptides and of **1** for the PTHR1 in the absence of G protein (the R0 state of the receptor) via an established competition assay.⁶³ **35** binds two-fold more strongly to the receptor R0 state than does **1**, while **22** binds two-fold *less* strongly than does **1** (Supporting Figure 9). This trend is consistent with the hypothesis that the low level of **35** detected in the mouse bloodstream after injection reflects peptide sequestration via receptor binding; however, it is not clear whether the four-fold affinity difference between **35** and **22** is sufficiently large to explain our observations. Another possible source of variation between these two α/β -peptides, which is not captured in the binding assay and was not tested, would involve the rate at which the agonist-PTHr1 complex is removed from the cell surface; the PTH(1-34)-PTHr1 complex is known to be rapidly internalized and to continue signaling from endosomes.⁶⁴ Overall, it appears that strong and rapid binding of **35** to the PTHR1, possibly augmented by unusually rapid receptor internalization, could explain how this α/β -peptide can exert a strong effect on Ca^{2+} levels despite the fact that **35** is barely detectable in the bloodstream at any point following injection.

There is a parallel between the *in vivo* behavior documented here for **35** and that reported previously for **27**.⁵⁷ Both of these molecules induce a robust calcemic effect upon injection in mice, but neither peptide achieves the bloodstream levels detected for **1** after injection. This parallel suggests that differences in the M-PTH sequence relative to the PTH sequence impart both strong calcium-mobilization activity and rapid clearance from the bloodstream. Collectively, our observations and previous data for PTHR1-agonist peptides show that there is no correlation between the strength of the calcemic effect induced by a PTHR1 agonist peptide and the duration of that peptide's persistence in the mouse bloodstream. These findings support the conclusion that monitoring the concentration of a PTHR1 agonist peptide in the mouse bloodstream does not necessarily provide a complete understanding of the peptide's pharmacokinetic behavior.

Extension of the optimal α/β substitution pattern to PTHrP(1-34) (**36**) and abaloparatide (**40**)

There are two known natural agonists of PTHR1, parathyroid hormone and parathyroid hormone-related protein (PTHrP).³⁵ As is the case with PTH, N-terminal segments of PTHrP, such as **36** or PTHrP(1-36) (**45**, see supporting figure 17 for structure), retain full agonist activity.⁶⁵ However, **1** and **45** differ considerably in their receptor-activating properties.⁶⁶ **1** induces long-lasting receptor activation in which cAMP generation continues after PTHR1 has been internalized; **1** remains bound to the internalized receptor.^{57, 64} In contrast, cAMP generation induced by **45** is short-lived and mostly limited to receptor molecules on the cell surface.^{63, 64} PTHR1 activated by **45** is only weakly active after internalization, at which point **45** has dissociated from the receptor.⁶⁶ Differences in the cAMP signaling profiles for PTH and PTHrP are attributed to a divergence in the capacity of these peptides to bind to the R0 state of PTHR1.^{57, 63, 64, 66, 67} **1** binds more tightly than does **45** to the R0 state of PTHR1, and **27** binds even more tightly than does **1**.^{57, 63, 67} This rank order coincides with the duration of PTHR1-mediated cAMP responses induced by

these α -peptides in cell-based assays.^{57, 63, 67–69} The mechanistic underpinnings of this correlation are an active area of investigation.^{64, 66, 70–74}

The distinctive signaling profile of PTHrP makes analogues of this peptide potentially advantageous as a basis for osteoporosis therapy, relative to **1**, because some PTHrP analogues can promote bone growth but are less prone to causing an undesirable rise in blood calcium ion levels (decreased calcemic effect).^{75–77} The decreased calcium mobilization activity of PTHrP analogues relative to **1** may be related to the weaker R0 affinity of such peptides.^{64, 78} The enhanced calcium mobilization activities of **27**⁵⁷, and a newly described PTHR1 agonist LA-PTH,^{69, 79} relative to **1**, along with the higher affinity of these peptides for the PTHR1 R0 state relative to **1**, support this hypothesis.

40 is a synthetic peptide based on the sequence of PTHrP(1-34) (**36**), containing multiple modifications in the segment spanning residues 22 to 31, that has recently completed a clinical trial for osteoporosis treatment with favorable results.^{68, 80, 81} **40** binds even more weakly than does **45** to the PTHR1 R0 state and also exhibits significantly reduced calcium mobilization activity relative to **1** in the clinic.^{68, 80} As is the case with teriparatide (i.e., **1**), **40** is delivered via subcutaneous injection.

Because the sequences of **36** and **40** differ significantly from those of **1** and **27**, we used the former two to evaluate the generality of the pattern of nine cyclic β residues in **35**. This substitution pattern enabled retention of PTHR1 agonist activity in the context of the **27** sequence, but these nine $\alpha \rightarrow$ cyclic β substitutions (to generate **26**) caused a substantial drop in potency in the context of the **1** sequence. Therefore, the outcome of these substitutions in the context of **36** and **40** could not be predicted (Figure 6).

Results of cell-based assays indicate that **36** is very similar to **1** in terms of stimulating cAMP production via engagement of the PTHR1 (Table 2, Supporting Figure 10), which is consistent with previous reports.⁶⁵ Imposing the nine $\alpha \rightarrow$ cyclic β replacements found in **35** on the sequence of **36**, to generate **39**, caused a small decline in agonist potency (~6-fold), which is very similar to the effect of these replacements in the sequence of **27** (Table 2). The nine $\alpha \rightarrow$ cyclic β replacements in the sequence of **40** caused a slightly larger decline in agonist potency (~18-fold), but the resulting α/β -peptide, **43**, was nevertheless quite potent in terms of stimulating cAMP production (Table 2). Incorporating only six $\alpha \rightarrow$ cyclic β replacements in the C-terminal regions of **36** and **40** sequences, at positions analogous to those of the replacements in **22**, provided analogues **38** and **42**, which were similar to one another in exhibiting potent agonist activity (Table 2, Supporting Figure 11). In contrast, when β^3 residues were used for the six $\alpha \rightarrow \beta$ replacements in the C-terminal region, the derivatives of **36** and **40** displayed different behaviors, with **37** only 4-fold less potent than **36** but **41** 200-fold less potent than **40**.

Variation in affinity for different PTHR1 conformational states among **1**, **36**, **40** and the α/β -peptide analogues offers another potential metric, beyond agonist activity (stimulation of cAMP production), by which peptides containing $\alpha \rightarrow \beta$ replacements might be compared to prototype α -peptides and to one another. We observed that **36** and **40** bound to the PTHR1 R0 conformational state⁶³ 3- and 22-fold less tightly than does **1**, respectively, in accord

with past findings (Supporting Figure 12).^{63, 68} The analogues of these peptides with a full set of $\alpha \rightarrow$ cyclic β replacements, **39** and **43**, bound to the PTHR1 R0 state 4- and 3-fold less tightly than does **1**, respectively. Thus, the incorporation of nine $\alpha \rightarrow$ cyclic β replacements into the PTHrP scaffold provides an analogue (**39**) that maintains the diminished PTHR1 R0 affinity characteristic of the prototype α -peptide, **36**, whereas the introduction of these same replacements in the scaffold of (**43**) modestly increases PTHR1 R0 affinity relative to the prototype. The origin of the different responses of the **36** and **40** sequences to the imposition of full sets of $\alpha \rightarrow$ cyclic β replacements, with respect to R0 affinity, is unknown, but could plausibly arise from altered modes of peptide interaction with the conformationally pliable receptor.

The PTHR1 bioactivity assay described above was used to evaluate the susceptibilities of **36**, **40**, **39** and **43** to degradation in SGF and SIF (Figure 7, Supporting Figure 13). Both of the α -peptides were very rapidly degraded in SGF, as expected, with no agonist activity detected for the **36** sample after 3 min exposure, and very little activity detected for the **40** sample at this time point (Figure 7). In contrast, no significant decline in agonist activity was detected for either α/β -peptide after 45 min exposure to SGF.

In SIF, the **36** sample displayed very low agonist activity after 10 min exposure, and the **40** sample displayed very low agonist activity after 20 min exposure (Supporting Figure 13). In each case, however, the analogous α/β -peptide manifested only a small decline in activity at the same time point. Thus, the pattern of $\alpha \rightarrow$ cyclic β replacements that generated an analogue of **27** with both potent agonist activity toward PTHR1 and substantial resistance to proteolysis seems to deliver the same favorable combination of properties when extended to the sequences of **36** and **40**.

DISCUSSION

The results described here represent a substantial advance in the development of backbone-modified polypeptide hormone analogues because we have shown that a systematic “ β -residue scan” can identify sites in the N-terminal region of **1** that tolerate $\alpha \rightarrow \beta$ replacements. Cyclic β residues prove to be superior to β^3 residues for maintaining potent agonist activity toward the PTHR1. The lack of high resolution structural information for PTHR1 prevents a detailed mechanistic interpretation of these findings, but this lack of structural insight also means that the successful outcome of the β scans could not have been predicted. The new, potent α/β -peptide agonists should be useful for refining models of the PTHR1 in the activated state.^{25–27}

We find that combining substitutions in the N-terminal region with an $\alpha \rightarrow \beta$ substitution pattern previously identified in the C-terminal region of **1** is most effective if cyclic β residues are employed throughout. Our approach ultimately identified nine sites of $\alpha \rightarrow$ cyclic β substitution that deliver a modestly potent PTHR1 agonist in the context of **1** and highly potent agonists in the context of three other sequences known to activate PTHR1, **27**, **36** and **40**. One of these potent α/β -peptide agonists, **35**, is active *in vivo*, inducing a calcemic effect in mice after injection. **35** is remarkably stable in environments that instantaneously destroy conventional peptides or α/β -peptides that contain long α segments

(e.g., **21**), such as kidney homogenate and simulated gastric fluid. These properties should support future efforts to achieve oral delivery of a bioactive α/β -peptide.

The observation that the analogues of **27**, **36** and **40** containing nine $\alpha \rightarrow \beta$ substitutions (**35**, **39** and **43**) manifest potent agonist activity, whereas the corresponding analogue of **1** (**26**) exhibits only moderate potency, suggests that different PTHR1 agonists interact with this receptor in subtly different ways. Since the six C-terminal $\alpha \rightarrow$ cyclic β substitutions used in this study are uniformly well-tolerated, with respect to retention of signaling activity, for all the PTHR1 agonist sequences tested (Table 1, Table 2, Supporting Figure 11), it is likely that the divergence in the impact of $\alpha \rightarrow \beta$ replacements on biological activity within different sequence contexts stems from modifications in the N-terminal portions of these peptides. The incorporation of an Ile5 $\rightarrow$ ACPC substitution in the sequence of **1** or related α/β -peptide analogues causes a substantial loss in agonist potency (Table 1), which is consistent with previous observations that modifications at position 5 of PTH analogues usually reduce agonist activity.⁵⁸ In this context, it is noteworthy that introduction of the Ile5 $\rightarrow$ ACPC substitution into the sequence of **27** does not impair agonist activity (Table 1). This finding is consistent with the previously documented capacity of the receptor affinity-enhancing α residue substitutions found in the M-PTH sequence to mask the detrimental effects of unfavorable modifications, such as C-terminal truncation.⁵⁷ Histidine is found instead of isoleucine at position 5 of the sequences of **36** and **40**. Past work has shown that His5 $\rightarrow$ Ile replacement in the PTHrP scaffold enhances PTHR1 affinity.⁶³ The hydrophobicity of the ACPC residue found at position 5 of **39** and **43** may enable this β residue to mimic the impact of isoleucine at this position of the all- α PTHrP/abaloparatide scaffold. It seems likely that characteristics of the residue at position 5 play a key role in controlling the activities of PTHR1 agonists.³⁵

Our results indicate that resistance to proteolysis and lifetime in the bloodstream are not correlated. For example, **35** is far less susceptible to proteolysis than is **1** under all conditions evaluated, yet **35** is only weakly detectable in the mouse bloodstream following injection, whereas **1** is robustly detected for a short period after injection (Fig. 5). We speculate that the low levels of **35** in the bloodstream, even immediately after injection, reflect rapid and essentially irreversible sequestration of this α/β -peptide by the PTHR1 in target tissues. Past work has shown that synthetic α -peptide PTHR1 agonists with high affinity for the R0 state of the PTHR1 are less evident in the bloodstream following injection than is **1**.^{57, 79} Nevertheless, these high-R0-affinity agonists induce more enduring biological responses than does **1**. This behavior may be related to the capacity of PTHR1 agonists with high R0 affinity to bind pseudo-irreversibly to the receptor, which is mostly located on bone and kidney cells, thereby removing peptide from circulation. Pseudo-irreversible binding requires the slow dissociation of ligand from receptor. Kinetics experiments have previously shown that **45** dissociates from PTHR1 more rapidly than does **1**,⁶³ and that PTH(1-28) dissociates more rapidly than does M-PTH(1-28),⁵⁷ which correlates with the affinity of these ligands for the R0 state of PTHR1. The high R0 affinity of **35** might therefore underlie the observation that this α/β -peptide is barely detectable in the bloodstream following injection. Differences in the capacity of **1** and **35** to induce receptor internalization may also contribute to variation in bloodstream concentrations of these peptides; however, few studies

have systematically evaluated structure-activity relationships for PTHR1 internalization. In one such study, significant differences in internalization efficacy were identified among various PTHR1 agonists, but none of the α/β -peptide analogues we developed has been evaluated in this way.⁶⁵

The relatively modest persistence of **22** in the mouse bloodstream after injection (only slightly greater than that **1**) contrasts with the much longer persistence observed previously for **21**.¹² The source of this *in vivo* difference between **21** and **22** is not clear, given the similar properties of these α/β -peptides in cell-based assays (Table 1). Differences in physicochemical properties might underlie variation in bloodstream persistence. Peptide **21** shows a larger retention time on C18 reversed-phase HPLC relative to **22**, which itself shows a larger retention time relative to **1**; this comparison indicates that the rank order of hydrophobicity is **21** > **22** > **1** (Supporting Figure 14). Enhanced peptide hydrophobicity can promote association with serum proteins, which can increase effective molecular weight and thereby slow filtration of the peptide from the bloodstream by kidney.⁸² Since kidney filtration represents a major route of PTH elimination from the bloodstream,⁶¹ differences between the bloodstream concentration profiles of **21** and **22** may be connected to differences in these molecules' hydrophobicities.

The successful introduction of $\alpha \rightarrow \beta$ replacements throughout the sequences of PTHR1 agonists with retention of potent bioactivity raises the possibility that these agonists might ultimately prove to be orally deliverable. The oral route is generally regarded as superior to all others in terms of patient preference, but this delivery strategy is problematic for peptides for at least two reasons. First, peptides are usually degraded rapidly in the stomach or small intestine. Second, peptides are too large to cross the wall of the small intestine passively and enter the bloodstream.⁴⁵ Results reported here show that the peptides **35**, **39**, and **43** overcome the first problem, and they lay the groundwork for future efforts to address the challenge of enabling large peptides to move from the gastrointestinal tract into the bloodstream. Most current attempts to solve this problem rely on formulation of a peptide with small molecules that promote the passive peptide diffusion through epithelial cells that line the intestinal lumen.⁴⁴ Favorable results have been reported for medium-size peptide drugs such as PTH derivatives,^{83–86} salmon calcitonin and glucagon-like peptide-1.⁴⁴ These efforts have relied on empirical screening efforts with large libraries of small molecule permeation enhancers and different dosing conditions using large populations of animals to identify optimal approaches.^{85, 87}

The results presented here strengthen the prospect that α/β -peptides will emerge as useful tools for analyzing biological phenomena at the molecular level, and perhaps even as therapeutic agents. Large-scale manufacture of α -peptides containing 20–40 residues for clinical use is feasible,⁸⁸ and the solid-phase synthesis of α/β -peptides is qualitatively similar to α -peptide synthesis. Ultimately, it would be attractive to produce α/β -peptides via the biosynthetic machinery, and recent reports encourage the hope that this goal could be achieved some day. Initial explorations suggested that the ribosomal biosynthesis of polypeptides containing β residues was inefficient or impossible.^{89–91} Subsequent findings, however, show that β -amino acids can be incorporated by the ribosome into polypeptides.

^{92–95} These findings raise the prospect that evolutionary approaches could be harnessed for discovery of new bioactive α/β -peptides, and for the production of optimized compounds.

EXPERIMENTAL

Peptide synthesis and purification

Peptides were synthesized as C-terminal amides on NovaPEG rink amide resin (EMD) using previously reported microwave-assisted solid-phase conditions, based on Fmoc protection of main chain amino groups⁴¹. Microwave-assisted reactions were carried out in a MARS multimode microwave reactor (CEM). Rink amide resin was weighed into a fritted polypropylene tube and allowed to swell first in DMF. For coupling of an activated amino acid to an unprotected amine on resin, the desired Fmoc-protected amino acid (125 μ mol) and HBTU (47 mg, 125 μ mol) were dissolved in 1.25 ml of 0.1 M HOBt in DMF (dimethylformamide). To the solution was added N,N-diethylisopropylamine (35 μ L, 200 μ mol). This mixture was vortexed briefly and allowed to react for at least 1 min. The activated amino acid solution was then added to the fritted polypropylene tube containing the resin. The resin was heated to 70°C in the microwave (2-min ramp to 70°C, 4-min hold at 70°C) with stirring. After the coupling reaction, the resin was removed from the microwave and washed with DMF (three times). For Fmoc deprotection, 3 ml of 20% piperidine in DMF was added to the resin, and the mixture was heated to 80°C in the microwave (2-min ramp to 80°C, 2-min hold at 80°C) with stirring. After the deprotection reaction, the resin was washed with DMF (three times). The cycles of coupling and deprotection were alternately repeated to give the desired full-length peptide. The resin was washed thoroughly (three times in DMF, three times in CH₂Cl₂) and then dried under a stream of air.

After synthesis, the peptides were cleaved from the resin, and side chains were deprotected using reagent K (82.5% TFA, 5% phenol, 5% H₂O, 5% thioanisole, 2.5% ethanedithiol) for two hours. The TFA solution was dripped into cold diethyl ether to precipitate the deprotected peptide. Peptides were purified on a prep-C18 column using reverse phase-HPLC. Purity was assessed by analytical RP-HPLC (solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile, C18 analytical column (4.6 $\times$ 250 mm), flow rate 1 mL/min, gradient 10–60% B solvent over 50 minutes). Masses were measured by MALDI-TOF-MS. All peptides exhibited >95% purity as evaluated using analytical reverse-phase C18 HPLC. See supporting figure 15 for analytical HPLC retention times, purity data and a list of observed masses from MALDI-TOF-MS (either monoisotopic [M+H]⁺, m/z or average [M+H]⁺, m/z, listed on the table). See supporting figure 16 for HPLC traces and MALDI-TOF mass spectra.

Protected β^3 -homoamino acids were purchased from PepTech or ChemImpex. Fmoc-ACPC was purchased from ChemImpex. Fmoc-APC(Boc) was synthesized through a reductive amination-functional group exchange protocol performed using a β -ketoester pyrrolidine analogue as previously described.⁵³ See supporting information for HPLC chromatograms, and mass spectrometry data.

Binding and cAMP assays

Reported IC₅₀ and EC₅₀ values are the average of 3 independent measurements, as indicated in figure captions. Data were fitted to a sigmoidal dose-response model with variable slope. Each assay consists of 4 data points (different concentrations) per peptide. Binding to the R0 conformation of the human PTHR1 was assessed by competition assays performed in 96-well plates by using membranes from either GP2.3 cells or Cos-7 cells transiently transfected to express human PTHR1. Binding was assessed by using ¹²⁵I-PTH(1–34) as tracer radio ligand and including GTPγS in the reaction (1×10^{-5} M).¹¹ Cell membranes were incubated with tracer ligand and varied concentrations of the indicated, unlabeled competitor peptide. Following incubation of membranes with peptides for > 1 hr, free peptide was separated from receptor bound peptide by filtration and the level of tracer peptide binding was evaluated by quantification of gamma radiation.

cAMP signaling was assessed using HEK-293-derived cell lines that stably express the Glosensor cAMP reporter (Promega Corp.)⁵¹ along with wild-type human PTHR1 (GP-2.3 cells) or human PTHR1 lacking the extracellular domain (GD5 cells). For cAMP dose-response assays, monolayers of confluent HEK 293 cells were pre-incubated with CO₂ independent media (Life Sciences) containing d-luciferin (0.5 mM) in 96 well plates at room temperature until a stable baseline level of luminescence was established (30 min). Varying concentrations of agonist were then added, and the time course of luminescence response was recorded using a Perkin Elmer plate reader following α/β-peptide addition. The maximal luminescence response (observed 12-20 min after ligand addition) was used for generating dose response curves.

In cases where peptides failed to induce cAMP responses near to the maximal response induced by **1**, a constraint was introduced when fitting of a sigmoidal dose response curve that specified that the maximal response for each peptide was above a threshold specified in the corresponding figure caption. This constraint enables an estimation of cAMP EC₅₀ values based on the assumption that these peptides are full agonists. Peptides that possess partial agonism properties are properly evaluated using this experimental approach.

Simulated gastric and intestinal fluid stability studies

Simulated gastric fluid was prepared according to instructions provided for test solutions in the United States Pharmacopeia. NaCl (20 mg) and pepsin from porcine mucosa (32 mg of >2500 U/mg pepsin, Sigma Aldrich P7012) were dissolved in 9.93 mL of deionized H₂O. Concentrated HCl (~37%) (700 μL) was then added to the solution to provide simulated gastric fluid (SGF). The resulting solution had a pH of ~1.2. SGF was prepared fresh for each assay and was incubated at 37° C before and during use.

All solutions used in the protocol, except peptide stocks, were first submitted to sonication to remove excess gas from the solution. Solutions of peptides (1 mM) dissolved in 10 mM acetic acid were used as stocks for these experiments. Aliquots of peptide stock were added to SGF (20 μL of peptide in 100 μL total solution, for a [peptide] of 200 μM) at t=0. Proteolysis was quenched at the indicated time by transferring 40 μL of the proteolysis solution into 80 μL of 10x PBS (1.37 M NaCl, 27 mM KCl, 80 mM Na₂HPO₄, 20 mM

KH_2PO_4 at pH 7.4), as pepsin is inactive at neutral pH. An additional 80 μL of deionized H_2O were added to the quenched solution to bring the total volume of the quenched solution to 200 μL ([peptide] in quenched solution was 40 μM). Each proteolysis experiment was run concurrently in duplicate to provide estimated variation (standard deviation) in the analyses described below. Control samples, in which proteolysis is prevented from occurring, were prepared by *first* mixing 32 μL of SGF with 80 μL of 10 $\times$ PBS to quench protease activity, followed by addition of 8 μL of peptide stock and 80 μL of deionized H_2O .

From these quenched solutions (either control or proteolysis groups), a portion (180 μL) was transferred to an HPLC vial for injection (injected 150 μL) onto an analytical RP-HPLC (see peptide synthesis and purification section above), and peaks were analyzed. The time course of peptide degradation was experimentally determined by integrating the area of the peak corresponding to the non-hydrolyzed peptide in a series of HPLC traces, with duplicate proteolysis reactions being used to generate error bars corresponding to the standard deviation. A small portion of the reaction solution (1 μL) was used to acquire MALDI-TOF mass spectrometry data for identification of peptide fragments resulting from proteolysis.

A portion of these quenched reaction solutions (or control solutions) was diluted 1:40 in CO_2 independent medium to provide 1 μM solutions for peptide bioactivity measurements. These solutions were then serially diluted (1:10) using CO_2 independent medium in a 96-well polypropylene dilution plate (concentration range 1000 nM to 1 nM on dilution plate). Aliquots from these serially diluted solutions (10 μL) were then transferred onto a different 96-well plate containing confluent monolayers of GP2.3 cells that had been preincubated with 90 μL of CO_2 independent media with 0.5 mM d-luciferin. The final peptide concentrations used in this assay ranged from 100 nM to 0.1 nM. Control experiments showed that quenched solutions of SGF without peptide did not induce luminescent responses in GP2.3 cells. Incubation of peptide in solutions of SGF, in which protease activity had been quenched prior to addition of peptide, for up to four hours, did not result in diminished peptide bioactivity relative to freshly prepared peptide stocks in CO_2 independent medium (data not shown). The luminescent responses were used to construct the dose response curves found in figure 4. Note that concentrations on the x-axis of figure 4 represent the estimated concentrations of the intact peptide before proteolysis. The concentration of intact peptide after proteolysis is not known for this assay.

Simulated intestinal fluid (SIF) was prepared according to instructions provided in the United States Pharmacopeia. KH_2PO_4 (68 mg) was dissolved in 2.5 mL of H_2O followed by the addition of 0.77 mL of 0.2 M NaOH. An additional 5 mL of H_2O was added to this solution followed by addition of 100 mg of porcine pancreatin (US Pharmacopeia grade, MP Bio #102557). The pH of the solution was adjusted to pH 6.8 through addition of 0.2 M NaOH. Deionized water was added to a final volume of 10 mL. This solution was prepared immediately before each assay and was incubated at 37° C before and during use.

Assessment of peptide stability was carried out as described for simulated gastric fluid with the following exceptions. Simulated intestinal fluid was used instead of simulated gastric fluid for all steps. Protease action was quenched through a 1:1 dilution of the proteolysis solution with a 50% solution (volume/volume) of CH_3CN in H_2O solution containing 1%

(by volume) trifluoroacetic acid (SIF quenching solution). Control solutions, in which the activity of SIF is quenched before addition of peptide, had the following composition: 40 μL SIF, 50 μL SIF quenching solution (mixed first), and 10 μL 1 mM peptide stock (100 μM [peptide] after quenching). HPLC and MALDI-TOF mass spectra analyses were not performed. Quenched solutions were diluted 1:100 into CO_2 independent medium on a polypropylene dilution plate, which was further diluted with three sequential 1:10 dilutions in CO_2 independent medium (concentrations of peptide on the dilution plate range from 1000 nM to 1 nM), as for experiments using SGF. Solutions from the dilution plate were diluted 1:10 onto the plate with GP2.3 cells to provide peptide concentrations ranging from 100 nM to 0.1 nM in the assay. Control experiments showed that quenched diluted solutions of SIF without peptide did not induce luminescence responses from GP2.3 cells and that solutions of SIF that were quenched before addition did not cause erosion of peptide bioactivity (data not shown). Note that concentrations on the x-axis of figure 4 represent the estimated concentrations of the intact peptide before proteolysis. The concentration of intact peptide after proteolysis is not known for this assay.

Rat kidney homogenate studies

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition; 2011). Adult female Sprague-Dawley rats (180–200 g; Harlan/Envigo, Indianapolis, IN, USA) were housed individually or in groups of two at room temperature under a 12-h light/dark cycle. Food and water were provided ad libitum. Animals were anesthetized with 1.5 g/kg urethane administered via intraperitoneal injections to effect. Body temperature of anesthetized animals was maintained at 37 °C using a homeothermic blanket (Harvard Apparatus). Anesthetized rats were perfused transcardially with 50 ml cold 0.01 M PBS (pH 7.4) and then decapitated. Kidney tissues were harvested, washed with ice-cold saline solution and adhering tissues were trimmed. 4 mL of incubation buffer (50 mM Tris-HCl, pH 7.4) was added per gram of tissue, which was homogenized using a Teflon homogenizer. The homogenates were centrifuged at 2000 g for 10 min at 4 °C and the supernatants were stored at –80°C for future analysis. The homogenate samples were used for the stability assay within 8 days of tissue collection. Bicinchoninic acid (BCA) assay was used to determine the total protein concentration in the tissue homogenates, which were then diluted to 2.5 mg protein/mL with incubation buffer.

PTH standards (**1** and **35**) were spiked in rat kidney homogenates to achieve a final concentration of 50 $\mu\text{g/mL}$ (~12 μM). Three replicates were prepared for each peptide. The spiked homogenates were incubated at 37 °C water bath and samples were collected at different time intervals (0, 1, 3, 5, 10, 15, 30, 45, 60, 90, 120 min). Two sets of samples were collected at each time point: a 50 μL sample was collected for liquid chromatography (LC) – mass spectrometry (MS) quantification study and a 10 μL sample was collected for matrix-assisted laser desorption/ionization (MALDI)-MS analysis. 5 μL internal standard (IS, 10 $\mu\text{g/mL}$ rat PTH) was spiked in each LC-MS sample as quality control and normalization factor. The collected samples were immediately mixed with equal volumes of 1% trifluoroacetic acid (TFA) to terminate protease activity. The sample mixtures were

centrifuged at 10,000 g for 10 minutes at 4 °C. The supernatants were dried in a speed vacuum concentrator and stored in –20 °C until further analysis.

Rat kidney homogenate studies: MALDI-MS analysis

A sample from each time point was reconstituted in 10 µL H₂O with 0.1% formic acid (FA). 1 µL reconstituted sample was mixed with 1 µL 2,5-dihydroxybenzoic acid matrix (DHB, 150 mg/mL dissolved in 49.95% methanol, 49.95% H₂O and 0.1% FA) and spotted onto a MALDI target plate. An ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with reflectron positive mode was used for all MALDI MS analysis. 5000 laser shots were accumulated for each sample at *m/z* 700-5000. The acquired data was analyzed with flexAnalysis 3.4 (Bruker Daltonics, Bremen, Germany).

Rat kidney homogenate studies: Liquid chromatography/mass spectrometry analysis

Sample from each time point was reconstituted in 50 µL H₂O with 0.1% formic acid (FA). Calibration standards (0, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50 and 75 µg/mL) were prepared by dissolving peptide standards in the same sample matrix (1:1 tissue homogenate:1% TFA in H₂O with 1 µg/mL rat PTH(1-34) as an internal standard).

A Dionex UltiMate 3000 UHPLC system (Thermo Scientific, Bremen, Germany) coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) were used for all LC/MS analysis. The analyte and IS were separated on a Phenomenex Kinetex C18 column (100 × 2.1 mm ID, 1.7 µm). Mobile phase A was H₂O with 0.1% FA and mobile phase B was acetonitrile with 0.1% FA. The following gradient was used to separate the analyte and IS (time/minute, % mobile phase B): (0, 15), (3.8, 50), (4, 98), (5.3, 98), (5.4, 15), (6, 15). The flow rate was set at 0.30 mL/min with column temperature at 30 °C and sampler temperature at 10 °C. Three injections were performed for each sample.

A multiplexed parallel reaction monitoring (PRM) method was set up on the mass spectrometer. Briefly, two scan events were defined. Scan event 1 was a full MS scan to monitor all ions from *m/z* 133.4 to 2000 with resolution of 70,000 (*m/z* 200). Scan event 2 was a targeted MS² event that multiplexed twice: +5 charged analyte and IS were fragmented together with normalized collisional energy (NCE) of 26 and +6 charged ions were fragmented together with NCE of 20.

Xcalibur (Thermo Scientific, Bremen, Germany) and Microsoft Excel were used for all data analysis. The *y*₃₂ ion peak area ratio of analyte to IS was calculated for each sample. A linear equation between concentrations and peak area ratios was established from calibration standards. The concentrations from unknown samples were calculated based on the peak area ratios and the linear equation.

Rat kidney homogenate studies: Bioassay analysis

The stability of peptides in kidney homogenate preparations was performed using a bioassay based readout as described above for simulated gastric fluid and simulated intestinal fluids, with the following exceptions. 1 mM stock solutions of peptide were diluted 1:85 into

kidney homogenate at $t = 0$ (~12 μM). The concentration of peptide in kidney homogenate following dilution approximates the concentration used in the LC/MS-based approach described above. Protease activity was quenched through a 1:1 dilution of the proteolysis reaction with SIF quenching solution at the indicated times (see above for the composition of this solution for the composition of SIF quenching solution). 10 μL of quenched protease solution was then mixed with 53 μL of CO_2 independent medium on the dilution plate. The concentration of peptide in the solutions provided after dilution into CO_2 independent medium is 1000 nM. This solution was serially diluted (three 1:10 dilutions) on the dilution plate (1000 nM to 1 nM), and 10 μL of solutions from the dilution plate were transferred to a plate with confluent monolayers of GP2.3 cells that had been preincubated in CO_2 independent medium containing 0.5 mM d-luciferin for 30 minutes. Note that concentrations on the x-axis of figure 3d represent the estimated concentrations of the intact peptide before proteolysis. The concentration of intact peptide after proteolysis is not known for this assay. Control solutions containing kidney homogenate without added PTHR1 agonist did not induce detectable luminescence in these assays.

In vivo pharmacology: calcemic response and pharmacokinetics

Mice (CD1, female, age 9–12 weeks, from Charles River) were treated in accordance with the ethical guidelines adopted by Massachusetts General Hospital as defined by the Institutional Animal Care and Use Committee. Mice were injected subcutaneously with vehicle (10 mM citric acid/150 mM NaCl/0.05% Tween-80, pH 5.0) containing **1** or α/β -peptide at a dose of 20 nmol/kg body weight using numbers of animals indicated in figure captions. Blood was withdrawn just prior to injection ($t = 0$) or at times thereafter. Tail vein blood was collected and immediately used for analysis. Blood Ca^{2+} concentration was measured with a Siemens RapidLab 348 Ca^{2+} /pH analyzer.

In vivo pharmacology: pharmacokinetics

Blood content of **1** or α/β -peptide derivatives was assessed in serum from mice ($n = 5$) injected with vehicle containing **1** or an α/β -peptide at a dose of 20 nmol/kg body weight in an experiment performed separately from the calcemic response assays described above. Blood was withdrawn just prior to injection ($t=0$) or at times thereafter. Tail vein blood was collected and treated with protease inhibitors (aprotinin, leupeptin, EDTA), centrifuged to remove blood cells, mixed with cAMP response assay buffer, and administered to GP2.3 cells. The raw luminescence readouts recorded in this assay were converted to blood peptide concentrations through use of a standard curve relating luminescence response to known peptide concentrations under identical assay conditions (supporting figure 8). Previous work has demonstrated this cAMP response-based method for quantifying the blood concentrations of PTHR1 agonists yields results similar to those from an ELISA-based method. Previous experiments showed GP2.3 cells exposed to plasma from mice injected with vehicle showed weak luminescence responses, indicating that observed cAMP responses were dependent on exogenous administration of a PTHR1 agonist.

Reversed-phase analytical HPLC retention time study

Peptides were injected onto a reversed phase analytical HPLC column using a 10-60% gradient of solvent B in solvent A, as described in the peptide synthesis section above. The retention time indicated corresponds to the time at which the maximal signal corresponding to the peptide of interest was observed. All peptides were injected consecutively on the same day using identical conditions. **1** was injected at the beginning and end of the studies to provide an estimation for variation in retention time resulting from alterations in instrument function. This variation is estimated to be less than 2 minutes.

Circular dichroism spectroscopy

Data collection was performed using an Aviv 420 Circular Dichroism Spectrophotometer. Wavelength scans were acquired at 20°C using a 1 nm step size and an averaging time of 3.0 sec from 260 to 195 nm. A 0.1 cm cell was used for all spectra. All samples were prepared at a peptide concentration of 50 μ M in 10 mM sodium phosphate buffer, pH 7.0. Peptide concentrations were calculated by measuring absorption at 280 nm after dissolving lyophilized peptide samples in sodium phosphate buffer (assuming ϵ (Trp)=5690 M⁻¹ cm⁻¹ at 280 nm).

Data Calculations

Data were processed by using the Microsoft Excel and GraphPad Prism 4.0 software packages. Data from binding and cAMP dose–response assays were analyzed using a sigmoidal dose–response model with variable slope. Data sets were statistically compared by using one-way ANOVA with Dunnett’s multiple comparison post-hoc test. Significance was assumed for $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Health grants GM-056414 (R01; S.H.G.), DK11794 (PO1; T.J.G.) and AR066261 (P30; T.J.G.). R.W.C. was supported in part by a Biotechnology Training Grant from NIGMS (T32 GM008349). Additional support was provided by the Wisconsin Alumni Research Foundation Discovery Challenge (R.W.C., B.C., N.K.), the University of Wisconsin-Madison Institute for Clinical and Translational Research and the Clinical and Translational Science Award program administered through the NIH National Center for Advancing Translational Sciences (NIH UL1TR000427 and KL2TR000428; R.G.T.), and the University of Wisconsin-Madison School of Pharmacy (N.K., R.G.T.). The LC/MS instrument was purchased through funding support from NIH S10 RR029531. L.L. acknowledges a Vilas Distinguished Achievement Professorship with funding provided by the WARF and University of Wisconsin-Madison School of Pharmacy. We thank the Analytical Instrumentation Center (School of Pharmacy, University of Wisconsin-Madison) for instrumentation support. We thank Thomas Dean (Massachusetts General Hospital) for excellent technical assistance.

ABBREVIATIONS

PTH1R	type-1 parathyroid hormone receptor
ACPC	(1S,2S)-2-aminocyclopentane carboxylic acid

APC	(3R,4S)-trans-4-aminopyrrolidine-3-carboxylic acid
SGF	simulated gastric fluid
SIF	simulated intestinal fluid

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		hPTHr1 EC ₅₀ , nM (Means ± SEM)	Derivative EC ₅₀ / PTH EC ₅₀ (nM)
1	SVSEIQLMH	0.09 ± 0.10	1.0
2	SVSEIQLMH	0.06 ± 0.08	0.7
3	SVSEIQLMH	0.29 ± 0.18	3.2
4	SVSEIQLMH	4.98 ± 0.03	55
5	SVSEIQLMH	9.84 ± 0.04	108
6	SVSEIQLMH	2.59 ± 0.05	28
7	SVSEIQLMH	14.57 ± 0.04	160
8	SVSEIQLMH	1.12 ± 0.05	12
9	SVSEIQLMH	18.69 ± 0.06	205
10	XVSEIQLMH	0.15 ± 0.15	1.6
11	SXSEIQLMH	0.27 ± 1.34	3.0
12	SVXEIQLMH	11.51 ± 0.11	126
13	SVSXQLMH	No activity	
14	SVSEXLQH	0.17 ± 0.08	1.9
15	SVSEIXLMH	0.34 ± 0.05	3.7
16	SVSEIQXMH	0.12 ± 0.05	1.3
17	SVSEIQLXH	9.74 ± 0.12	107
18	SVSEIQLMZ	No activity

Figure 1.

Sequences of PTH(1-34) analogues in the N-terminal β-scan library and cAMP stimulating potency. Each peptide is comprised of the native sequence for α- residues for positions 10-34 (see Figure 2 for full sequence). The single letter code is used to indicate conventional proteinogenic amino acids. Colored dots indicate sites at which α-to-β replacements have been introduced. β³-Residues display an identical sidechain to that displayed by the corresponding α-residue. cAMP inducing potencies (EC₅₀) are shown for analogues with cells expressing hPTHr1. Average EC₅₀ values are from values obtained in three

independent experiments for all α/β -peptides or six independent experiments for PTH(1-34). Analogues with “no activity” have too weak of activity to be characterized using this method. Full dose-response curves are found in Supporting Figure 1.

Figure 2. Sequences of a peptides containing α -to- β replacements in the N-terminal segment, C-terminal segment or both

Differences between the PTH(1-34) and M-PTH(1-34) scaffolds are highlighted in bold in the M-PTH(1-34) sequence. The single letter code is used to indicate conventional proteinogenic amino acids. Colored dots indicate sites at which α -to- β replacements have been introduced. β^3 -Residues display an identical sidechain to that displayed by the corresponding α -residue. Note that selected derivatives contain an α -amino isobutyric acid residue (Aib) at position 3.

Figure 3. Stability of PTH(1-34) or M-Cyc-9β in rat kidney homogenate

Peptides were incubated in kidney homogenates from a single, female Sprague-Dawley rat for the indicated durations, and intact peptide concentration was evaluated using a liquid chromatography/mass spectrometry (LC/MS) approach or a bioassay readout as described in the methods section. Sites of protease-catalyzed hydrolysis were evaluated using MALDI-TOF mass spectrometry. (a) The concentration of intact peptide from LC/MS analysis as a function of duration of incubation. Data points represent mean $\pm$ standard deviation from two replicates. The curve results from fitting of the data to an exponential decay function, with half-lives listed. (b-c) Identification of peptide fragments derived from PTH(1-34) (b)

or **M-Cyc-9 β** (**c**) by MALDI-TOF mass spectrometry as a function of time for a single experiment. Peptide-derived fragments that may give rise to observed signals are inserted above the peak of interest (**d**) Peptide bioactivity following exposure to kidney homogenate was assessed using a biological assay with two replicates. Data points represent mean $\pm$ SEM. Times listed after peptide names represent the duration of time that the peptide was incubated with kidney homogenate before quenching of protease activity. Curves result from fitting of a sigmoidal dose-response model with variable slope.

Figure 4. Degradation of PTH(1-34) derivatives in SGF and SIF

Peptide bioactivity following exposure to SGF (simulated gastric fluid) was assessed using a biological assay (panels **a-d**). Data points represent mean $\pm$ SEM from two technical replicates within a single experiment. Independent experiments (Supporting Figure 7) provide similar results. Curves result from fitting of a sigmoidal dose response model with variable slope to the data. Note that the times listed after peptide names represent the duration of time that the peptide was incubated with in SGF before quenching of protease activity. (**e**) Sites of proteolysis in PTH(1-34) and derivatives SGF. Sites of protease action were identified by analyzing crude proteolysis mixtures using MALDI-TOF mass

spectrometry as described in the methods section from a single experiment. Arrows point to major sites of proteolytic cleavage See Figure 2 for structures. **(f-g)** Bioassay to assess degradation of PTH(1-34) or **M-Cyc-9 β** in simulated intestinal fluid (SIF). The sigmoidal dose response model did not provide a well fitted curve for SIF experiments and was thus omitted in panels **f** and **g**. See the methods section for additional details. Data points represent mean $\pm$ SEM from two technical replicates for a single experiment.

Figure 5. Properties of PTH(1-34) and α/β -peptide derivatives in mice

Peptides were injected at a dose of 20 nmol/kg by subcutaneous injection in CD1 mice, $n = 5$, female, 8-12 weeks of age. * $p < 0.05$ vs. vehicle as assessed by one-way ANOVA with Dunnett's multiple comparison test. Vehicle is 0.05% Tween-80, 10 mM citrate, 150 mM NaCl (pH 5.0). Data points represent mean $\pm$ SEM from the five distinct mice per treatment group. Data points are connected with lines to guide the eye; these lines have no independent meaning. (a) PTH(1-34) and derivatives induce a calcemic response following subcutaneous injection into mice (b) Bloodstream bioavailability time course for PTH(1-34) or selected α/β -peptide derivatives. A luminescence response was induced in GP2.3 cells following addition of 2 μ L of serum (mixed with CO₂ independent medium) taken from mice at different time points after injection (x-axis). This raw luminescence response was converted to the concentration of peptide in the bloodstream through use of a standard curve relating peptide concentrations to luminescence responses. See Supporting Figure 8 for the raw luminescence responses induced by serum and standard curves.

Figure 6.

A comparison of the structures of highly substituted analogues of PTH, PTHrP and Abalopartide. The segment in which Abalopartide differs from PTHrP(1-34) is highlighted in red lettering.

Figure 7. Degradation of PTHrP and Abaloparatide analogues in SGF

Peptide bioactivity following exposure to SGF was assessed using a biological assay. Data points represent mean $\pm$ SEM from two technical replicates in a single experiment. Curves result from fitting of a sigmoidal dose response model with variable slope to the data. Note that the times listed after peptide names represent the duration of time that the peptide was incubated with in SGF before quenching of protease activity.

Table 1
Summary of the cAMP inducing properties of α/β -peptide analogues of PTH(1-34)

Tabulation of receptor activation for the derivatives in Figure 2 with hPTHr1. Mean EC₅₀ values are averaged from EC₅₀ values obtained in three independent experiments for all α/β -peptides or six independent experiments for PTH(1-34). See Supporting Figure 3 for cAMP dose-response curves and Supporting Figure 9 for composite binding curves. See methods section for experimental details.

Peptide	hPTHr1 EC ₅₀ , nM (Means $\pm$ SEM)	Derivative EC ₅₀ /PTH(1-34) EC ₅₀
1	0.08 $\pm$ 0.01	1.0
19	0.14 $\pm$ 0.03	1.7
20	3.0 $\pm$ 0.56	36.7
21	0.17 $\pm$ 0.05	2.1
22	0.26 $\pm$ 0.05	3.2
23	1.84 $\pm$ 0.11	22.6
24	0.71 $\pm$ 0.04	8.7
25	167 $\pm$ 6.3	2,050
26	19 $\pm$ 1.3	234
27	0.34 $\pm$ 0.04	4.2
28	0.52 $\pm$ 0.19	6.3
29	0.40 $\pm$ 0.11	4.9
30	0.44 $\pm$ 0.10	5.4
31	0.51 $\pm$ 0.15	6.3
32	0.83 $\pm$ 0.07	10
33	1.8 $\pm$ 0.23	22
34	0.79 $\pm$ 0.25	9.7
35	0.78 $\pm$ 0.12	9.5

Table 2
Summary of the cAMP inducing properties of α/β -peptide analogues of PTHrP and Abaloparatide

Tabulation of receptor activation and binding properties for the derivatives in Figure 6 with hPTHrP1. Mean EC₅₀ values are averaged from EC₅₀ values obtained in the number of independent experiments indicated (*n*). See Supporting Figure 12 for composite binding curves for selected analogues and Supporting Figures 10 and 12 for composite dose-response cAMP induction curves. See methods section for experimental details. The data found in this figure are derived from experiments distinct from those that provide data for Table 1.

Derivative	hPTHrP1 cAMP EC ₅₀ , nM (mean $\pm$ SEM)	<i>n</i>	Derivative EC ₅₀ /PTH(1-34) EC ₅₀
1	0.07 $\pm$ 0.01	8	1.0
35	0.48 $\pm$ 0.12	3	7.1
36	0.08 $\pm$ 0.02	4	1.2
37	0.32 $\pm$ 0.15	2	4.7
38	0.65 $\pm$ 0.19	2	9.4
39	0.51 $\pm$ 0.16	5	7.4
40	0.02 $\pm$ 0.005	7	0.3
41	4.13 $\pm$ 0.27	2	60.4
42	0.18 $\pm$ 0.01	2	2.6
43	0.36 $\pm$ 0.09	4	5.3