

Use of Backbone Modification To Enlarge the Spatiotemporal Diversity of Parathyroid Hormone Receptor-1 Signaling via Biased Agonism

Shi Liu,[†] Frederic G. Jean-Alphonse,[‡] Alex D. White,^{‡,§} Denise Wootten,[⊥] Patrick M. Sexton,[⊥] Thomas J. Gardella,^{*,||} Jean-Pierre Vilardaga,^{*,‡,⊥} and Samuel H. Gellman^{*,†,⊥}

[†]Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

[‡]Department of Pharmacology and Chemical Biology, and [§]Graduate Program in Molecular Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, United States

^{||}Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

[⊥]Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, VIC 3052, Australia

Supporting Information

ABSTRACT: The type-1 parathyroid hormone receptor (PTHr1), which regulates calcium homeostasis and tissue development, has two native agonists, parathyroid hormone (PTH) and PTH-related protein (PTHrP). PTH forms a complex with the PTHr1 that is rapidly internalized and induces prolonged cAMP production from endosomes. In contrast, PTHrP induces only transient cAMP production, which primarily arises from receptors on the cell surface. We show that backbone modification of PTH(1–34)-NH₂ and abaloparatide (a PTHrP derivative) with a single homologous β -amino acid residue can generate biased agonists that induce prolonged cAMP production from receptors at the cell surface. This unique spatiotemporal profile could be useful for distinguishing effects associated with the duration of cAMP production from effects associated with the site of cAMP production.

Information transfer at the molecular level is critical in living systems, and many natural messages are encoded in amino acid sequences. Evolutionary modulation of these messages is focused on side chains of proteins and peptides. Chemical synthesis enables an expansion in polypeptide language via backbone modification, which can be implemented without changing side chains. We have begun to explore this approach in the context of polypeptide hormones that activate B family GPCRs, many of which are targets of therapeutic interest.^{1–4} Previously, β -amino acids have been used to modulate proteolytic stability, receptor-subtype selectivity, and signaling profiles of peptidic GPCR agonists.^{5–9} The present study introduces a new capability of $\alpha \rightarrow \beta$ substitution, modulation of the spatiotemporal characteristics of signal transduction.

Agonist binding to a GPCR induces conformational changes that are sensed by cytosolic binding partners. Different intracellular partners, such as G_s or β -arrestins, are predicted to engage distinct receptor conformations.¹⁰ Biased agonism¹¹ is observed when a ligand favors a receptor conformation attractive to one partner relative to others. Our group

previously discovered that replacing multiple α amino acid residues of glucagon-like peptide-1 (GLP-1) with cyclic β -amino acid residues generated biased agonists favoring receptor interaction with β -arrestins relative to G_s. However, these α/β -peptides were less potent agonists than the original α -peptide (GLP-1).⁹ Here we show that minimal backbone alteration enabled by the use of homologous β -residues can generate potent agonists with high signaling selectivity at the PTHr1 and an altered spatiotemporal signaling profile relative to α -peptide prototypes.

The PTHr1 is an important target for the treatment of osteoporosis and hypoparathyroidism. Activation of the PTHr1 by its two native ligands, PTH and PTHrP, initiates G_s/cAMP/PKA signaling and β arrestin recruitment.^{12,13} PTH and PTHrP differ, however, in their spatiotemporal signaling profiles.¹⁴ PTHrP causes transient cAMP production from receptors on the cell surface, while PTH triggers prolonged cAMP production after the receptor–agonist complex has translocated to endosomes.^{15–17}

Signaling duration is an important determinant of the physiological effects exerted by PTHr1 agonists,¹⁸ but the subcellular location of GPCR signaling can also influence physiological outcome.¹⁹ Therefore, molecular tools that enable dissection of the spatial and temporal dimensions of PTHr1 signaling would be useful for elucidating the physiological roles of this receptor.^{20,21} We asked whether backbone modification could provide a PTHr1 agonist with a unique spatiotemporal signaling profile relative to natural hormones, specifically, an agonist that would induce prolonged receptor activation that occurs only at the cell surface.

Because binding of β -arrestin to the PTHr1 is necessary for receptor internalization and attenuates cell-surface G_s/cAMP signaling,²² PTHr1 engaged by a G_s-biased agonist might persist at the cell surface and cause prolonged cAMP production from that location, if the agonist had sufficient affinity. Previous approaches to G_s-biased agonists of the PTHr1 have involved amino acid side chain modification and

Received: April 17, 2019

Published: September 9, 2019



achieved only limited success.^{13,23,24} We pursued $\alpha \rightarrow \beta$ backbone modification^{5,8,25,26} with PTH(1–34) and with PTHrP analogue abaloparotide (ABL), both of which are used to treat osteoporosis.^{27,28}

The N-terminal region of peptidic PTHR1 agonists inserts deeply into the transmembrane domain (PTHR1-TMD) and causes conformational changes to which transducers respond.²⁹ The PTHR1 conformation required for G_s activation appears to differ from that needed for recruitment of β -arrestins.³⁰ We hypothesized that backbone modification in the N-terminal region of PTH(1–34) might induce PTHR1 conformations that differentially affect G_s activation and β -arrestin recruitment. Our search for G_s -biased agonists based on PTH(1–34)-NH₂ began with a set of previously described analogues generated by replacing each of the first eight residues, individually, with the (S^*)- β^3 , (S^*)- β^2 , or (R^*)- β^2 homologue (Figure 1; S^* and R^* defined in the Supporting

PTH (1–34) : SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-NH₂
 Peptide 1: SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-NH₂
 Peptide 2: SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-NH₂
 Peptide 3: SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-NH₂

ABL: AVSEHQLLHDKGKSIQDLRRRELLEKLLUKLHTA-NH₂
 Peptide 4: AVSEHQLLHDKGKSIQDLRRRELLEKLLUKLHTA-NH₂
 Peptide 5: AVSEHQLLHDKGKSIQDLRRRELLEKLLUKLHTA-NH₂
 Peptide 6: AVSEHQLLHDKGKSIQDLRRRELLEKLLUKLHTA-NH₂

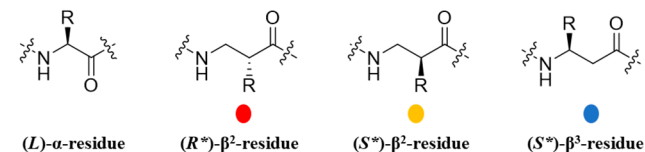
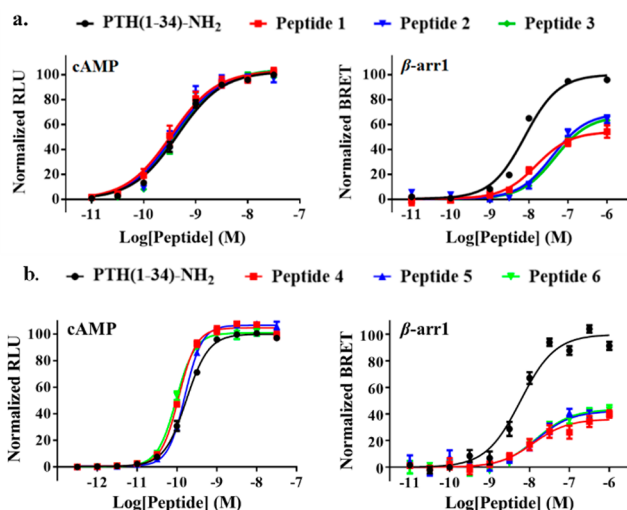


Figure 1. α/β PTHR1 agonists displaying significant G_s -biased agonism relative to PTH(1–34)-NH₂. See the Supporting Information for the definition of R^* and S^* . "L" denotes norleucine. U denotes 2-aminoisobutyric acid.

Information).⁸ Several of these backbone-modified analogues (Figure S1) were indistinguishable from PTH(1–34)-NH₂ in stimulating cAMP production, a marker of G_s activation, as evaluated in HEK-293 cells stably expressing human PTHR1 and the GloSensor for cAMP detection (Table S1). In new studies, we evaluated these analogues for their ability to recruit β -arrestins to the PTHR1 via bioluminescence resonance energy transfer (BRET) measurements using CHO-FlpIn cells stably transfected with PTHR1-Rluc8 and β -arrestin-1-Venus or β -arrestin-2-Venus (Table S1). Considerable variation in recruitment of β -arrestins to the PTHR1 was observed. Incorporating the (S^*)- β^2 homologue at position 5 or the (R^*)- β^2 homologue at position 7 or 8 (peptides 1–3) caused the most significant BRET reductions (Figure 2a and S6a).

With PTH(1–34)-NH₂ as the reference agonist, we quantified the signaling bias of 1–3 based on the Black–Leff operational model (Figure 2c), in which $\Delta \log(\tau/K_A)$ denotes the bias factor.^{31,32} This quantification method minimizes the dependence of bias factor on cell type.³¹ Both 2 and 3 were ~ 10 -fold biased toward the G_s -mediated signaling pathway vs β -arrestin recruitment, relative to PTH(1–34)-NH₂. Peptide 1 displayed a more moderate bias. We found that previously described peptides containing a single side-chain modification,²⁴ Tyr¹-PTH(1–34)-NH₂ and Trp¹-PTHrP(1–36)-NH₂, are defective for β -arrestin recruitment, as reported; however, both were ~ 5 -fold less potent than PTH(1–34)-NH₂ in



c.

Peptide	$\Delta \log(\tau/K_A)_{cAMP/\beta arr1}$	$\Delta \log(\tau/K_A)_{cAMP/\beta arr2}$
PTH (1-34)-NH ₂	0	0
1	0.70* \pm 0.18	0.57# \pm 0.22
2	1.02* \pm 0.10	1.06* \pm 0.11
3	1.03* \pm 0.11	0.96* \pm 0.11
4	1.02* \pm 0.12	1.26* \pm 0.24
5	0.88* \pm 0.14	1.14* \pm 0.14
6	1.23* \pm 0.15	1.15* \pm 0.10

Figure 2. Activity profiles of PTH and α/β G_s -biased PTHR1 agonists. (a, b) Concentration–response effects of PTH and peptides 1–6 for cAMP production and β -arrestin-1 recruitment (β -arrestin-2 data in SI). Data represent mean \pm s.e.m. from $n \geq 6$ independent experiments. (c) Calculated bias factors of 1–6 relative to PTH(1–34)-NH₂. *, #Statistically significant difference from PTH using one-way ANOVA (* $P < 0.0001$; # $P < 0.001$).

stimulating cAMP production (Figure S2). Neither of these side chain-modified peptides displayed the level of signal bias observed for backbone-modified peptides 1–3.

ABL shows a significant bias toward cAMP production relative to PTH(1–34)-NH₂ (Figure S3). Despite this bias, ABL is relatively effective at recruiting β -arrestins to the PTHR1 (Figure S4), which motivated us to use ABL to evaluate the generality of the $\alpha \rightarrow \beta$ modification strategy. Initial modifications of ABL involved positions 7 and 8, based on observations with PTH(1–34)-NH₂ analogues. However, most of these modifications caused substantial declines in stimulation of cAMP production (Figure S5). The differential tolerance of β homologue incorporation at positions 7 and 8 suggests that the PTHR1-TMD-binding modes of PTH(1–34)-NH₂ and ABL are different, which is consistent with the discovery that the N-terminal regions of PTH(1–34) and PTHrP(1–34) are not functionally equivalent for receptor binding.³³ More interesting results emerged from $\alpha \rightarrow \beta$ substitutions at positions 1 and 2 of ABL. Peptides 4–6 were very effective at stimulating cAMP production but significantly less effective than ABL or PTH(1–34)-NH₂ at recruiting β -arrestins (Figures 2b, S6b and S7).

The signaling duration of G_s -biased agonists 1–6 was evaluated with “washout” assays in which cells were stimulated with an agonist for a defined period, and then the agonist was washed away, and cAMP production was monitored (“ligand-off phase”).^{34,35} For 2, 3, and 5, cAMP production was more

transient than for PTH(1–34)-NH₂ (Figure S8). In contrast, **1** induced more prolonged signaling relative to PTH(1–34)-NH₂ (Figure 3a). While ABL was more short-acting than

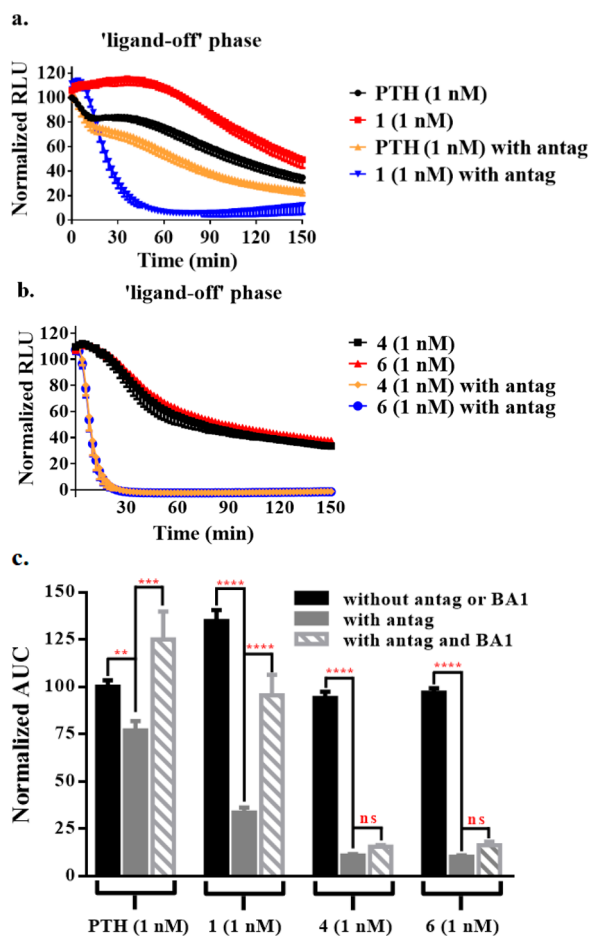


Figure 3. Effect of competitive antagonist and BA1 on signal duration of PTHR1 agonists. (a, b) Postwashout cAMP production mediated by 1 nM PTH, **1**, **4**, or **6** in HEK293 cells stably expressing hPTHR1. (c) Washout assay data from part a and b presented as area under curve (AUC); data for measurements with BA1 are included. Data represent mean \pm s.e.m. of $n = 4$ (PTH(1–34)-NH₂ and **1**), $n = 3$ (**4** and **6**) independent experiments. All the ligand activities are normalized to that of PTH(1–34)-NH₂ at 1 nM. Statistically significant difference using one-way ANOVA (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$).

PTH(1–34)-NH₂ at their equiactive concentrations,³⁶ both **4** and **6** performed similarly to PTH(1–34)-NH₂ in this assay at the tested concentration (Figures 3b and S9).

To evaluate the location of the cAMP production stimulated by **1**, **4**, or **6**, we introduced an excess of PTHR1 antagonist (D)Trp¹²,Tyr³⁴-bPTH(7–34) during the washout assay. We hypothesized that this peptidic antagonist should compete with agonist molecules for PTHR1 remaining on the cell surface, but the antagonist should not reach internalized agonist–receptor complexes.^{22,37,38} For **1**, **4**, and **6**, the presence of antagonist after washout caused a profound drop in the duration and extent of cAMP production (Figures 3 and S10). In contrast, only a modest drop was observed after washout with antagonist for PTH(1–34)-NH₂, which is known to continue stimulating cAMP production from endosomes.¹⁵ These observations support the conclusion that most cAMP

production induced by **1**, **4**, or **6** involves PTHR1 at the cell surface.

Both the PTHR1 and β -arrestin are overexpressed in the cells used for the BRET assay, while the washout assay was performed in HEK293 cells expressing β -arrestin at endogenous levels. We therefore evaluated β -arrestin recruitment induced by **1**, **4**, or **6** via coimmunoprecipitation (co-IP) of endogenous β -arrestin and the PTHR1 in HEK293 cells stably expressing hemagglutinin (HA)-tagged PTHR1.¹³ As shown in Figures 4 and S11, PTH(1–34)-NH₂ effectively induced co-IP

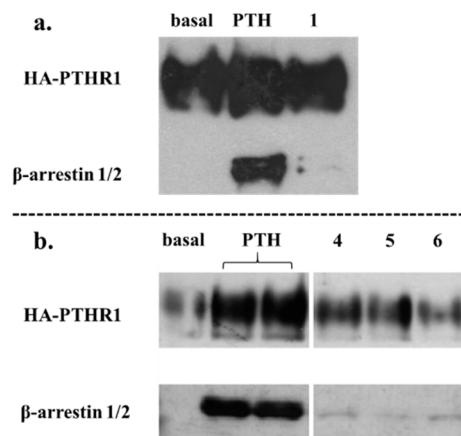


Figure 4. Recruitment of endogenous β -arrestin to PTHR1 detected by coimmunoprecipitation. HEK293 cells stably expressing HA-tagged PTHR1 were challenged with PTH(1–34)-NH₂ or peptides **1**–**6**. The left and right panels of b are from the same blot with unrelated data omitted from the center

of the PTHR1 and β -arrestin, while agonists **1**, **4**, and **6** induced very little co-IP. Peptide **6** was further studied via confocal microscopy in HEK293 cells expressing SNAP-tagged PTHR1 and GFP-tagged β -arrestin. Peptide **6** did not induce observable β -arrestin recruitment to the PTHR1, while PTH(1–34)-NH₂ effectively triggered the colocalization of the PTHR1 and β -arrestin (Figure S12). These observations support the conclusion that biased agonists **1**, **4**, and **6** are relatively ineffective at inducing β -arrestin recruitment to the PTHR1, which causes receptors activated by these peptides to remain at the cell surface.

Blocking endosomal acidification was shown to prolong cAMP production of PTH.¹⁷ We therefore conducted additional washout experiments with bafilomycin A1 (BA1), an inhibitor of endosomal acidification,³⁹ to gain further insight into the signaling profiles of **1**, **4**, and **6**. After agonist washout, cells were incubated in a solution containing both BA1 and the antagonist. Inclusion of BA1 after washout had very little effect on the extent of postwashout cAMP production induced by **4** or **6** (Figures 3c and S13a), but BA1 caused a substantial increase in postwashout cAMP production induced by **1**, relative to the level observed with the antagonist alone. This observation suggests that the single $\alpha \rightarrow \beta^2$ replacement at position 5 of PTH(1–34)-NH₂ (to generate **1**) exerts dual effects on the agonist profile by diminishing β -arrestin recruitment induced by **1** and rendering **1** susceptible to acidification-induced dissociation from the receptor within endosomes. These two effects could be synergistic in restricting the cAMP production induced by **1** to receptors on the cell surface in the absence of BA1, although the

endosomal dissociation effect is presumably minor. When tested at the same ligand concentration, ABL also displayed more sustained postwashout cAMP production in the presence of BA1 (Figure S13b), suggesting that endosomal acidification induces ABL dissociation from the receptor. In contrast to the agonist activity of **1** or ABL, the spatial restriction of cAMP production induced by **4** or **6** seems to arise predominantly from their inability to induce receptor internalization.

We have shown that introducing a single extra CH₂ unit into the backbone of a long polypeptide agonist can substantially change the signaling profile that results from engagement of a class B GPCR. Moreover, altering the position of the extra CH₂ along the backbone can lead to different signaling outcomes. (In the SI, we interpret the behavior of our biased agonists in terms of recent advances in GPCR structural analysis.) Because this backbone-focused approach maintains the native side chain sequence, and because the backbone-modified peptides can be prepared via conventional solid-phase synthesis, designing and generating a “test set” of β -containing analogues based on a known polypeptide agonist is straightforward (in contrast to exploring a potentially infinite set of analogues containing unnatural side chains⁴⁰). While displaying high potency and efficacy in stimulating cAMP production via activation of the PTHR1, β -residue-containing peptides **1**, **4**, and **6** are highly defective for β -arrestin recruitment. When compared to the α -peptide prototypes at equiactive concentrations, these three α/β -peptides induce a longer duration of cAMP production that emanates predominantly from receptors at the cell surface. There is a long-standing belief that sustained cAMP production from PTHR1 stimulation will lead to prolonged RANKL activation and bone resorption.⁴¹ However, it is unclear at present whether the spatial aspect of cAMP production contributes to the RANKL activation. The highly G_s-biased agonists **4** and **6** could be useful tools to explore questions of this type.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b04179.

Protocols for peptide synthesis, functional cell assays, coimmunoprecipitation, and confocal microscopy, method for the quantification of bias factors, and analytical data for all the peptides (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: gardella@helix.mgh.harvard.edu.

*E-mail: jpv@pitt.edu.

*E-mail: gellman@chem.wisc.edu.

ORCID

Shi Liu: 0000-0002-9113-6207

Patrick M. Sexton: 0000-0001-8902-2473

Jean-Pierre Vilardaga: 0000-0002-1217-1435

Samuel H. Gellman: 0000-0001-5617-0058

Notes

The authors declare the following competing financial interest(s): S.H.G. is a cofounder of Longevity Biotech, Inc., which is pursuing biomedical applications of alpha/beta-peptides.

■ ACKNOWLEDGMENTS

This research was supported in part by NIH Grant R01 GM056414 (to S.H.G.), R01 DK111427 (to J.P.V.), R01 DK116780 (to J.P.V.), T32-GM008424 (to A.D.W.), P01 DK11794 (to T.J.G.), and P30 AR066261 (to T.J.G.).

■ REFERENCES

- (1) Broichhagen, J.; Podewin, T.; Meyer-Berg, H.; von Ohlen, Y.; Johnston, N. R.; Jones, B. J.; Bloom, S. R.; Rutter, G. A.; Hoffmann-Roder, A.; Hodson, D. J.; Trauner, D. Optical Control of Insulin Secretion Using an Incretin Switch. *Angew. Chem., Int. Ed.* **2015**, *54*, 15565–15569.
- (2) Fremaux, J.; Venin, C.; Mauran, L.; Zimmer, R. H.; Guichard, G.; Goudreau, S. R. Peptide-oligourea hybrids analogue of GLP-1 with improved action in vivo. *Nat. Commun.* **2019**, *10*, 924.
- (3) Kroll, C.; Mansi, R.; Braun, F.; Dobitz, S.; Maecke, H. R.; Wennemers, H. Hybrid Bombesin Analogues: Combining an Agonist and an Antagonist in Defined Distances for Optimized Tumor Targeting. *J. Am. Chem. Soc.* **2013**, *135*, 16793–16796.
- (4) Liu, Y. T.; Cai, Y. Y.; Liu, W.; Li, X. H.; Rhoades, E.; Yan, E. C. Y. Triblock peptide-linker-lipid molecular design improves potency of peptide ligands targeting family B G protein-coupled receptors. *Chem. Commun.* **2015**, *51*, 6157–6160.
- (5) Cheloha, R. W.; Maeda, A.; Dean, T.; Gardella, T. J.; Gellman, S. H. Backbone modification of a polypeptide drug alters duration of action in vivo. *Nat. Biotechnol.* **2014**, *32*, 653–655.
- (6) Cheloha, R. W.; Chen, B. M.; Kumar, N. N.; Watanabe, T.; Thorne, R. G.; Li, L. J.; Gardella, T. J.; Gellman, S. H. Development of Potent, Protease-Resistant Agonists of the Parathyroid Hormone Receptor with Broad beta Residue Distribution. *J. Med. Chem.* **2017**, *60*, 8816–8833.
- (7) Johnson, L. M.; Barrick, S.; Hager, M. V.; McFedries, A.; Homan, E. A.; Rabaglia, M. E.; Keller, M. P.; Attie, A. D.; Saghatelian, A.; Bisello, A.; Gellman, S. H. A Potent alpha/beta-Peptide Analogue of GLP-1 with Prolonged Action in Vivo. *J. Am. Chem. Soc.* **2014**, *136*, 12848–12851.
- (8) Liu, S.; Cheloha, R. W.; Watanabe, T.; Gardella, T. J.; Gellman, S. H. Receptor selectivity from minimal backbone modification of a polypeptide agonist. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 12383–12388.
- (9) Hager, M. V.; Johnson, L. M.; Wootten, D.; Sexton, P. M.; Gellman, S. H. beta-Arrestin-Biased Agonists of the GLP-1 Receptor from beta-Amino Acid Residue Incorporation into GLP-1 Analogues. *J. Am. Chem. Soc.* **2016**, *138*, 14970–14979.
- (10) Mary, S.; Damian, M.; Louet, M.; Floquet, N.; Fehrentz, J. A.; Marie, J.; Martinez, J.; Baneres, J. L. Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 8304–8309.
- (11) Rajagopal, S.; Rajagopal, K.; Lefkowitz, R. J. Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat. Rev. Drug Discovery* **2010**, *9*, 373–386.
- (12) Abousamra, A. B.; Juppner, H.; Force, T.; Freeman, M. W.; Kong, X. F.; Schipani, E.; Urena, P.; Richards, J.; Bonventre, J. V.; Potts, J. T.; Kronenberg, H. M.; Segre, G. V. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat os-teoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 2732–2736.
- (13) Gesty-Palmer, D.; Chen, M. Y.; Reiter, E.; Ahn, S.; Nelson, C. D.; Wang, S. T.; Eckhardt, A. E.; Cowan, C. L.; Spurney, R. F.; Luttrell, L. M.; Lefkowitz, R. J. Distinct beta-arrestin- and G protein-independent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J. Biol. Chem.* **2006**, *281*, 10856–10864.
- (14) Cheloha, R. W.; Gellman, S. H.; Vilardaga, J. P.; Gardella, T. J. PTH receptor-1 signalling-mechanistic insights and therapeutic prospects. *Nat. Rev. Endocrinol.* **2015**, *11*, 712–724.

- (15) Ferrandon, S.; Feinstein, T. N.; Castro, M.; Wang, B.; Bouley, R.; Potts, J. T.; Gardella, T. J.; Vilaradaga, J. P. Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat. Chem. Biol.* **2009**, *5*, 734–742.
- (16) Vilaradaga, J. P.; Jean-Alphonse, F. G.; Gardella, T. J. Endosomal generation of cAMP in GPCR signaling. *Nat. Chem. Biol.* **2014**, *10*, 700–706.
- (17) Gidon, A.; Al-Bataineh, M. M.; Jean-Alphonse, F. G.; Stevenson, H. P.; Watanabe, T.; Louet, C.; Khatri, A.; Calero, G.; Pastor-Soler, N. M.; Gardella, T. J.; Vilaradaga, J. P. Endosomal GPCR signaling turned off by negative feedback actions of PKA and v-ATPase. *Nat. Chem. Biol.* **2014**, *10*, 707–709.
- (18) Okazaki, M.; Ferrandon, S.; Vilaradaga, J. P.; Bouxsein, M. L.; Potts, J. T.; Gardella, T. J. Prolonged signaling at the parathyroid hormone receptor by peptide ligands targeted to a specific receptor conformation. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 16525–16530.
- (19) Tsvetanova, N. G.; von Zastrow, M. Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. *Nat. Chem. Biol.* **2014**, *10*, 1061–1065.
- (20) Horwitz, M. J.; Tedesco, M. B.; Sereika, S. M.; Syed, M. A.; Garcia-Ocana, A.; Bisello, A.; Hollis, B. W.; Rosen, C. J.; Wysolmerski, J. J.; Dann, P.; Gundberg, C.; Stewart, A. F. Continuous PTH and PTHrP infusion causes suppression of bone formation and discordant effects on 1,25(OH)(2) vitamin D. *J. Bone Miner. Res.* **2005**, *20*, 1792–1803.
- (21) Ricarte, F. R.; Le Henaff, C.; Kolupaeva, V. G.; Gardella, T. J.; Partridge, N. C. Parathyroid hormone(1–34) and its analogs differentially modulate osteoblastic Rankl expression via PKA/SIK2/SIK3 and PP1/PP2A-CRTRC3 signaling. *J. Biol. Chem.* **2018**, *293*, 20200–20213.
- (22) Ferrari, S. L.; Behar, V.; Chorev, M.; Rosenblatt, M.; Bisello, A. Endocytosis of ligand-human parathyroid hormone receptor 1 complexes is protein kinase C-dependent and involves beta-arrestin-2-Real-time monitoring by fluorescence microscopy. *J. Biol. Chem.* **1999**, *274*, 29968–29975.
- (23) Bisello, A.; Chorev, M.; Rosenblatt, M.; Monticelli, L.; Mierke, D. F.; Ferrari, S. L. Selective ligand-induced stabilization of active and desensitized parathyroid hormone type 1 receptor conformations. *J. Biol. Chem.* **2002**, *277*, 38524–38530.
- (24) Cupp, M. E.; Nayak, S. K.; Adem, A. S.; Thomsen, W. J. Parathyroid Hormone (PTH) and PTH-Related Peptide Domains Contributing to Activation of Different PTH Receptor-Mediated Signaling Pathways. *J. Pharmacol. Exp. Ther.* **2013**, *345*, 404–418.
- (25) Peggion, E.; Mammi, S.; Schievano, E.; Silvestri, L.; Schiebler, L.; Bisello, A.; Rosenblatt, M.; Chorev, M. Structure-function studies of analogues of parathyroid hormone (PTH)-1–34 containing beta-amino acid residues in positions 11–13. *Biochemistry* **2002**, *41*, 8162–8175.
- (26) Schievano, E.; Mammi, S.; Carretta, E.; Fiori, N.; Corich, M.; Bisello, A.; Rosenblatt, M.; Chorev, M.; Peggion, E. Conformational and biological characterization of human parathyroid hormone hPTH(1–34) analogues containing beta-amino acid residues in positions 17–19. *Biopolymers* **2003**, *70*, 534–547.
- (27) Berg, C.; Neumeyer, K.; Kirkpatrick, P. Teriparatide. *Nat. Rev. Drug Discovery* **2003**, *2*, 257.
- (28) Shirley, M. Abaloparatide: First Global Approval. *Drugs* **2017**, *77*, 1363–1368.
- (29) Zhao, L. H.; Ma, S. S.; Sutkeviciute, I.; Shen, D. D.; Zhou, X. E.; de Waal, P. W.; Li, C. Y.; Kang, Y. Y.; Clark, L. J.; Jean-Alphonse, F. G.; White, A. D.; Yang, D. H.; Dai, A. T.; Cai, X. Q.; Chen, J.; Li, C.; Jiang, Y.; Watanabe, T.; Gardella, T. J.; Melcher, K.; Wang, M. W.; Vilaradaga, J. P.; Xu, H. E.; Zhang, Y. Structure and dynamics of the active human parathyroid hormone receptor-1. *Science* **2019**, *364*, 148–153.
- (30) Vilaradaga, J. P.; Frank, M.; Krasel, C.; Dees, C.; Nissenson, R. A.; Lohse, M. J. Differential conformational requirements for activation of G proteins and the regulatory proteins arrestin and G protein-coupled receptor kinase in the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein. *J. Biol. Chem.* **2001**, *276*, 33435–33443.
- (31) Kenakin, T.; Watson, C.; Muniz-Medina, V.; Christopoulos, A.; Novick, S. A Simple Method for Quantifying Functional Selectivity and Agonist Bias. *ACS Chem. Neurosci.* **2012**, *3*, 193–203.
- (32) Black, J. W.; Leff, P. Operational Models of Pharmacological Agonism. *Proc. R. Soc. London, B, Biol. Sci.* **1983**, *220*, 141–162.
- (33) Gardella, T. J.; Luck, M. D.; Wilson, A. K.; Keutmann, H. T.; Nussbaum, S. R.; Potts, J. T.; Kronenberg, H. M. Parathyroid Hormone (PTH)-PTH-related Peptide Hybrid Peptides Reveal Functional Interactions between the 1–14 and 15–34 Domains of the Ligand. *J. Biol. Chem.* **1995**, *270*, 6584–6588.
- (34) Dean, T.; Vilaradaga, J. P.; Potts, J. T.; Gardella, T. J. Altered selectivity of parathyroid hormone (PTH) and PTH-Related protein (PTHrP) for distinct conformations of the PTH/PTHrP receptor. *Mol. Endocrinol.* **2008**, *22*, 156–166.
- (35) Tamura, T.; Noda, H.; Joyashiki, E.; Hoshino, M.; Watanabe, T.; Kinoshita, M.; Nishimura, Y.; Esaki, T.; Ogawa, K.; Miyake, T.; Arai, S.; Shimizu, M.; Kitamura, H.; Sato, H.; Kawabe, Y., Identification of an orally active small-molecule PTHR1 agonist for the treatment of hypoparathyroidism. *Nat. Commun.* **2016**, *7*, DOI: 10.1038/ncomms13384.
- (36) Hattersley, G.; Dean, T.; Corbin, B. A.; Bahar, H.; Gardella, T. J. Binding Selectivity of Abaloparatide for PTH-Type-1-Receptor Conformations and Effects on Downstream Signaling. *Endocrinology* **2016**, *157*, 141–149.
- (37) Goldman, M. E.; McKee, R. L.; Caulfield, M. P.; Reagan, J. E.; Levy, J. J.; Gay, C. T.; Dehaven, P. A.; Rosenblatt, M.; Chorev, M. A new highly potent parathyroid-hormone antagonist: [D-Trp12, Tyr34]-bPTH-(7–34)NH₂. *Endocrinology* **1988**, *123*, 2597–2599.
- (38) Thomsen, A. R. B.; Plouffe, B.; Cahill, T. J.; Shukla, A. K.; Tarrasch, J. T.; Dosey, A. M.; Kahsai, A. W.; Strachan, R. T.; Pani, B.; Mahoney, J. P.; Huang, L. Y.; Breton, B.; Heydenreich, F. M.; Sunahara, R. K.; Skiniotis, G.; Bouvier, M.; Lefkowitz, R. J. GPCR-G Protein-beta-Arrestin Super-Complex Mediates Sustained G Protein Signaling. *Cell* **2016**, *166*, 907–919.
- (39) Yoshimori, T.; Yamamoto, A.; Moriyama, Y.; Futai, M.; Tashiro, Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.* **1991**, *266*, 17707–17712.
- (40) Zhang, H. K.; Sturchler, E.; Zhu, J.; Nieto, A.; Cistrone, P. A.; Xie, J.; He, L. L.; Yea, K.; Jones, T.; Turn, R.; Di Stefano, P. S.; Griffin, P. R.; Dawson, P. E.; McDonald, P. H.; Lerner, R. A. Autocrine selection of a GLP-1R G-protein biased agonist with potent antidiabetic effects. *Nat. Commun.* **2015**, *6*, 8918.
- (41) Qin, L.; Raggatt, L. J.; Partridge, N. C. Parathyroid hormone: a double-edged sword for bone metabolism. *Trends Endocrinol. Metab.* **2004**, *15*, 60–65.