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Title Page

Parathyroid Hormone (PTH) and PTH-Related Peptide Domains Contributing to Activation of Different PTH Receptor-Mediated Signaling Pathways

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Running Title: Domains of PTH and PTHrP mediating PTH1R signaling

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List of nonstandard abbreviations: AA amino acid; AFU arbitrary fluorescence units; ALU arbitrary luminescent units; CHO Chinese hamster ovary (cells); DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-

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related kinase; GPCR, G protein-coupled receptor; hPTH1R, human PTH1R; IP_1, inositol monophosphate; PBS, phosphate-buffered solution; PKA, protein kinase A; PKC, protein kinase C; PLCβ, phospholipase Cβ; PLD, phospholipase D; PTH, parathyroid hormone; PTH1R, PTH1 receptor; PTHrP, PTH-related peptide; RANKL, receptor activator of nuclear factor kappa-B ligand; RH, relative humidity; RhoA, Ras homolog gene family, member A; SEM, standard error of mean; TRF, time resolved fluorescence

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ABSTRACT

Parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP), acting through the osteoblast PTH1R, play important roles in bone remodeling. Intermittent administration of PTH(1-34) (teriparatide) leads to bone formation while continuous administration paradoxically leads to bone resorption. Activation of PTH1R promotes regulation of multiple signaling pathways, including G_s/cAMP/PKA, G_q/calcium/PKC, β-arrestin recruitment and ERK1/2 phosphorylation as well as receptor internalization but their role in promoting anabolic and catabolic actions of PTH(1-34) are unclear. In the present investigation, a collection of PTH(1-34) and PTHrP(1-34) peptide analogs were evaluated in orthogonal hPTH1R functional assays capturing G_s- and G_q-signaling, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization to further define the patterns of PTH1R signaling they stimulate and further establish peptide domains contributing to agonist activity. Results indicate both N- and C-terminal domains of PTH and PTHrP are critical for activation of signaling pathways. However, modifications of both regions leads to more substantial decreases in agonist potency and efficacy to stimulate G_q-signaling, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization than to stimulate G_s-signaling. The substantial contribution of the peptide C-terminal domain in activation hPTH1R signaling suggests a role in positioning of the peptide N-terminal region into the receptor J-domain. Several PTH and PTHrP peptides evaluated in this study promote different patterns of biased agonist signaling and may serve as useful tools to further elucidate therapeutically relevant PTH1R signaling in osteoblasts. With a better understanding of therapeutically

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relevant signaling, novel biased peptides with desired signaling could be designed for safer and more effective treatment of osteoporosis.

INTRODUCTION

Parathyroid hormone (PTH(1-84)) and parathyroid hormone-related peptide (PTHrP(1-141)), acting through the parathyroid hormone receptor (PTH1R1), play important roles in facilitating calcium homeostasis and bone remodeling (Fitzpatrick and Bilezikian, 1996). PTH1R is a class B GPCR family receptor possessing a long extracellular N-terminus important for hormone binding and an intracellular 130 AA C-terminus containing protein interaction domains for cytosolic adaptor and effector proteins regulating receptor expression and signaling (Vilardaga et al., 2010). The N-terminal 34 AAs of both peptides contain the minimal sequence required for PTH1R activation and signal transduction (Gensure et al., 2005). Mechanisms accounting for PTH and PTHrP binding and activation of PTH1R have been widely investigated and support a two-site model involving initial rapid binding of the peptide C-terminal region (AAs 23-34) to the PTH1R N-terminus, followed by a slower interaction of the peptide N-terminus (AAs 1-14) with the J-domain containing transmembrane α-helices and interconnecting extracellular loops (Gardella et al., 2001; Gensure et al., 2005; Castro et al., 2005). Activation of PTH1R leads to regulation of multiple signaling pathways, including G_s/adenylyl cyclase/cAMP/PKA- (Abou-Samra et al., 1992), $G_{q/11}$/phospholipase Cβ/calcium/PKC- (Abou-Samra et al., 1992), and $G_{12/13}$/RhoA/PLD-signaling (Wang and Stern, 2010), as well as G-protein-dependent and G-protein-independent and β-arrestin-dependent activation of ERK1/2 (Syme et al., 2005; Gesty-Palmer et al., 2006). PTH1R-mediated activation of PKC also occurs through a PLCβ-independent mechanism (Takasu et al., 1999).

PTH(1-34) (teriparatide) is the only anabolic drug on the US market for treatment

of osteoporosis (Kawai et al., 2011). Although intermittent administration of PTH(1–34) promotes bone formation and reduces the incidence of fracture, continuous administration promotes bone resorption and hypercalcemia (Tam et al., 1982; Hock et al., 1992; Dempster et al., 1993). Anabolic effects of intermittent PTH(1-34) are due to PTH1R-mediated increases in osteoblast number, resulting from combined enhancement of preosteoblast differentiation and reduction of apoptosis of mature osteoblasts (Jilka, 2007). The catabolic effect of continuous PTH(1-34) is due to increased expression of osteoblast RANKL and decreased expression of the RANKL decoy protein osteoprotegerin, leading to enhanced osteoclastogenesis (Ma et al., 2001). The contribution of osteoblast PTH1R signaling pathways to these divergent actions of PTH(1-34) are unclear and requires further elucidation.

Biased agonists are widely described for GPCRs and are characterized by the capacity to activate only part of the total repertoire of signaling activated by endogenous ligand due to stabilization of distinct active receptor conformations coupling to activation of discrete signaling pathways (Kenakin, 2004; 2007; Urban et al., 2007). Several PTH and PTHrP analogs have been previously described that promote PTH1R differential signaling, including [Trp^1]PTHrP(1-36) and [Bpa^1]PTHrP(1-36) reported to activate hPTH1R-mediated G_s- and not G_q-signaling, β-arrestin recruitment, ERK1/2 phosphorylation or receptor internalization (Bisello et al., 2002, Gesty-Palmer et al., 2006; 2009). Similarly, [Gly^1,Arg^{19}]PTH(1-28) is reported to stimulate G_s- and not G_q-signaling (Takasu et al., 1999; Yang et al., 2007). In addition, [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 is a reported β-arrestin biased agonist promoting β-arrestin-dependent ERK1/2 activation and an inverse agonist for G_s-signaling (Gesty-Palmer et al., 2006; 2009). *In*

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vivo studies of biased PTH peptides on bone formation suggest G_s-signaling plays a predominant role while G_q-signaling plays little or no role (Rixon et al., 1994; Hilliker et al., 1996; Whitfield et al., 1996; Yang et al., 2007). Furthermore, a recent report suggests that PTH1R-mediated $G_{q/11}$ signaling plays a role in restraining the anabolic action of intermittent PTH(1-34) (Ogata et al., 2011). However, others report that PTH1R-mediated G_q-signaling also contributes to anabolic effects of intermittently administered PTH(1-34) (Rhee et al., 2006; Murrills et al., 2004; Guo et al., 2010). In addition, β-arrestin has been reported to be required for mediating intermittent PTH1R-mediated bone formation without enhancing osteoclastogenesis in wild type mice, but not in mice with β-arrestin ablated (Ferrari et al., 2005; Pierroz et al., 2009; Getsy-Palmer et al., 2009). Furthermore, [D-Trp^{12},Tyr^{34}]PTH(7-34) has been reported to mediate intermittent PTH(1-34) bone formation found absent in β-arrestin knockout mice suggesting that β-arrestin signaling is sufficient (Gesty-Palmer et al., 2009). In light of these different conclusions, the exact PTH1signaling pathways participating in bone reformation require further elucidation.

In this study, several PTH and PTHrP peptide analogs were tested in orthogonal hPTH1R assays capturing G_s- and G_q-signaling, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization to comprehensively characterize domains of PTH and PTHrP peptides contributing to activation of different signaling pathways and further establish patterns of differential signaling promoted. Information from this investigation should help guide design of additional biased PTH1R agonists to serve as valuable tools to further elucidate of therapeutically relevant PTH1R-mediated signaling.

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Furthermore, this information could help to guide design of peptide analogs with better therapeutic profiles for treatment of osteoporosis.

MATERIALS AND METHODS

Materials.

Human PTH(1-34) (H-4835), PTHrP(1-34) (H-6630), PTH(1-37) (H-5974), PTHrP(1-37) (H-5494), PTH(1-31) (H-2274), PTH(1-31)NH_2 (H-3408), [Cyclo(Glu^{22}-Lys^{26}),Leu^{27}]PTH(1-31)NH_2 (H-4058), [Tyr^1]PTH(1-34) (H-3092), [$Nle^{8,18}$, Tyr^{34}]PTH(1-34) (H-9110), PTH(2-38) (H-1316), PTHrP(1-16) (H-6575), PTHrP(7-34)NH_2 (H-9100), and [Asn^{10},Leu^{11},D-Trp^{12}]PTHrP(7-36)NH_2 (H3274) were purchased from Bachem (San Diego, CA). Bovine PTH(3-34) (H-3088), [$Nle^{8,18}$,Tyr^{34}]PTH(3-34)NH_2 (N-1130), [Tyr^{34}]PTH(7-34)NH_2 (N-1110), [$Nle^{8,18}$,Tyr^{34}]PTH(7-34)NH_2 (H-9120], and [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 (H-9115) were purchased from Bachem (San Diego, CA). PTH(7-34) (HOR-266) was purchased from Prospec (East Brunswick, NJ). Human MPTH(1-28), ([$Aib^{1,3}$, Gln^{10}, Har^{11}, Ala^{12}, Trp^{14}, Arg^{19}]PTH(1-28)) and [Gly^1,Arg^{19})PTH(1-28) were purchased from Anaspec (Fremont, CA). Human PTH(1-28), [Trp^1]PTHrP(1-36) [Bpa^1]PTHrP(1-36), MPTH(1-14) ([$Aib^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2), ZP2307 ([AC_5C^1-Aib^3,Leu^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Asp^{17}]PTH(1-17)NH_2 and [$Aib^{1,3}$, Nle^8,Gln^{10},Har^{11}]PTH(1-11)NH_2 were all synthesized using standard peptide synthesis and purification methods. Specifically, molecular identities of the synthesized peptides were determined using high-resolution mass spectrometry. Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) hybrid mass spectrometer (MALDI-TOF-TOF) were employed to confirm synthetic peptides were assembled correctly. All the mass spectra showed accurate molecular weight matching the expected or the theoretical molecular weight calculated based on the chemical formula of the peptides.

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The purity and the homogeneity of the synthetic peptides was determined using analytical reversed-phase high-pressure liquid chromatography (RP-HPLC) performed using a C18 column. The semi-preparative and analytical chromatograms from the RP-HPLC runs confirmed purity of >90% for the synthetic peptides. Analytical data are shown in the **Supplement**.

cAMP accumulation assay. PTH1R agonist-mediated stimulation of intracellular cAMP accumulation was measured using the competitive immunoassay Hunter^{TM} eXpress cAMP assay kit based upon a β-galactosidase enzyme complementation platform (DiscoveRX, Fremont, CA) (Olsen and Eglen, 2007). Briefly, 100 μl aliquots of thawed CHO-K1 cells expressing recombinant hPTH1R were added to 96-well microtiter plates (30,000 cells/well), and plates were incubated at 37^{o}C (5% CO_2, 95% relative humidity (RH)) for 24-48 hr before performing the assay. Test peptides dissolved in 100% DMSO were diluted to desired concentrations with cell assay buffer and added to appropriate wells followed by incubation of assay plates for 30 min at 37^{o}C (5% CO_2, 95% RH). The rest of the assay was performed according to the manufacturer's specifications and the chemiluminescent signal was measured using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA). Data are expressed in arbitrary luminescence units (ALU). The average basal signal was 5,000 ALU and the average signal obtained with a maximal concentration of PTH(1-34) was 69,000 ALU.

Inositol phosphate accumulation assay. hPTH1R-mediated stimulation of inositol monophosphate (IP_1) accumulation was measured using the CisBio IPOne^{TM} TRF (time resolved fluorescence) assay kit (Bedford, MA) (Trinquet et al., 2011) and using the same CHO-K1 clone stably expressing recombinant hPTH1R used for cAMP accumulation

assays (DiscoveRX, Fremont, CA). Briefly, 100 µl aliquots of cells (30,000 cells/well) were added to 96-well assay microtiter plates and incubated for 48 hr at 37^{o}C (5% CO_2, 95% RH). Cell media (65 µl) was then removed, 35 µl of test peptide originally solubilized in 100% DMSO and diluted to the appropriate concentration with stimulation buffer was added to appropriate wells, assay plates were incubated for 90 min at 37^{o}C (5% CO_2, 95% RH) and cells were lysed for 2 hr with lysis buffer containing antibodies. A total of 70 µl of lysed cells was transferred to a half volume white 96-well microtiter plate, and fluorescent signal was measured using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA) at wavelengths of 665 nm and 620 nm. Data are expressed as arbitrary fluorescence units (AFU). The average blank was 16000 AFU and the average signal in the presence of a maximal concentration of PTH(1-34) was 32,000 AFU.

β-arrestin-2 recruitment assay. PTH1R-mediated stimulation of β-arrestin-2 recruitment was measured directly using the Pathfinder^{TM} assay kit (DiscoveRX, Fremont, CA). This assay is based upon β-galactosidase enzyme complementation technology in which β-arrestin-2 is fused to a N-terminal deletion mutant of β-galactosidase (EA protein) and hPTH1R is fused to a smaller weakly binding 42 AA complementing Prolink fragment. Agonist activation of the receptor promotes direct interaction with β-arrestin-2 and the hPTH1R and complementation of the two β-galactosidase fragments to form functional enzyme (Bassoni et al., 2012; McGuinness et al., 2009; Yin et al., 2009). Briefly, vials of frozen CHO-K1 cells stably expressing recombinant hPTH1R were thawed in a 37^{o}C water bath for 10 sec and diluted in pre-warmed media, 100 µl aliquots of cell suspension were added to 96-well assay microtiter plates (8,000 cells/well) and plates were incubated for 48 hr at 37^{o}C (5% CO_2, 95% RH)

before performing assays. Test peptide solubilized in 100% DMSO and diluted to the desired concentrations with cell media was added to appropriate wells and plates were incubated for 90 min at 37^{o}C (5% CO_2, 95% RH). The rest of the assay was performed according to the manufacturer's specifications and the chemiluminescent signal was measured using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as ALU and the average blank was 32,000 ALU and the average signal in the presence of a maximal concentration of PTH(1-34) was 650,000 ALU.

ERK1/2 phosphorylation assay. The CisBio Cellul'erk^{TM} TRF assay kit (Bedford, MA) was used to measure PTH1R-mediated ERK1/2 phosphorylation in the same CHO-K1 clone stably expressing hPTH1R (DiscoveRX, Fremont CA) used for cAMP and IP_1 accumulation assays. This assay is based upon a competitive sandwich ELISA platform using an anti-phospho-ERK1/2 antibody labeled with a TRF acceptor d2 fluorophore and an anti-ERK1/2 antibody labeled with TRF donor Eu^{3+}-cryptate fluorophore. Briefly, 50 μl aliquots of cells (15,000 cells/well) were added to 96-well assay microtiter plates and incubated overnight at 37^{o}C (5% CO2, 95% RH), cell media was removed and cells were incubated with PBS for 20 min at room temperature. Test peptide solubilized in 100% DMSO and diluted to the desired concentration with PBS was added to appropriate wells, assay plates were incubated for 15 min at room temperature and then cells were lysed for 30 min. The remainder of the assay was performed according to the manufacturer's specifications, fluorescent signal was measured at wavelengths 665 nm and 620 nm using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA) and data are expressed as AFU. The average blank was 6,000 AFU and the average signal in the presence of a maximal concentration of PTH(1-34) was 10,000 AFU.

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hPTH1R internalization assay. Agonist stimulation of PTH1R endocytosis into early endosomes was measured using the DiscoveRX PathHunter™ Total Internalization eXpress assay kit (DiscoveRX, Fremont, CA) based upon a β-galactosidase enzyme complementation platform (Olson and Eglen, 2007). Specifically, when U2OS cells stably expressing hPTH1R tagged with the Prolink interact with EA-tagged endosomes during receptor internalization and enzyme complementation occurs leading to generation of a chemiluminescent product. This assay measures both β-arrestin dependent and independent receptor internalization. Briefly, vials of frozen U2OS cells stably expressing hPTH1R were thawed in a 37°C water bath for 30 sec, diluted in pre-warmed media, 100 µl aliquots of cell suspension were added to 96-well assay microtiter plates (8,333 cells/well) and plates were incubated for 24 hr at 37°C (5% CO_2, 95% RH). Test peptide solubilized in 100% DMSO and diluted to desired concentrations with cell assay buffer were added to appropriate wells containing media and plates were incubated for 3 hr at 37°C (5% CO_2, 95% RH). The rest of the assay was performed according to the manufacturers specifications and chemiluminescent signal was measured using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as ALU and the average blank was 300,000 ALU and the average signal in the presence of a maximal concentration of PTH(1-34) was 130,000 ALU.

Data analysis. Agonist EC_{50} and antagonist IC_{50} values were determined by fitting dose response data to a three-parameter sigmoidal function using a nonlinear least squares curve fitting program (Prism, GraphPad, San Diego, CA) and the data for the time course of PTH(1-34) and PTHrP(1-34) stimulation of β-arrestin recruitment were fit to a hyperbolic function using the same curve fitting program. The statistical significance

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of differences between EC_{50} values obtained for PTH(1-34) and other PTH and PTHrP peptides ($P < 0.001$) in the same hPTH1R functional assay was determined using an unpaired two-tailed students t-test with Prism and the statistical significance of differences in efficacy promoted by different peptide analogs (% of the maximal response by the highest concentration of PTH(1-34)) ($P < 0.05$) were determined using a paired one-tail students t-test with Prism. The statistical significance of differences in EC_{50} values and efficacy values ($P < 0.05$) for a given test peptide obtained in PTH1R functional assays was determined using a one-way ANOVA analysis followed by a Tukey's post-test using Prism. The correlation between agonist activity (E_{max}/EC_{50}) of various PTH and PTHrP peptide analogs obtained in different hPTH1R functional assays and data were subjected to Deming (Mode II) linear regression analysis using Prism. Ligand bias (β factor) was quantified by a method described by Rajagopal et al. (2011) in which the equiactive comparison method was employed. Using this approach, the concentrations of agonist required for an equiactive response in one signaling pathway (pathway 1) and another signaling pathway (pathway 2) are extrapolated from the three-parameter sigmoidal curve fit of the concentration-response data (Rajagopal et al., 2011). A bias factor representing the relative stabilization of one signaling pathway over another is given by the equation:

$$\beta = \log \left(\left(\frac{E_{max,1}}{EC_{50,1}} \frac{EC_{50,2}}{E_{max,2}} \right)_{lig} X \left(\frac{E_{max,2}}{EC_{50,2}} \frac{EC_{50,1}}{E_{max,1}} \right)_{ref} \right)$$

The statistical significance of differences in calculated β factors for different peptide analogs ($P < 0.05$) was determined using a one-way ANOVA analysis followed by a Tukey post-test using Prism.

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RESULTS

PTH/PTHrP peptide analog stimulation of PTH1R-mediated G_s-signaling. Agonist activation of hPTH1R receptor expressed in different cells commonly results in G_s coupling, stimulation of adenylyl cyclase and increases of intracellular cAMP followed by stimulation of PKA and phosphorylation of select intracellular substrates. Measurement of PTH and PTHrP peptide analog stimulation of PTH1R-mediated cAMP accumulation in a CHO-K1 cell clone stably expressing recombinant hPTH1R was accomplished using the DiscoveRX Hunter^{TM} eXpress ELISA-based enzyme complementation cAMP assay. Representative concentration-response curves for PTH(1-34), [Tyr^1]PTH(1-34), PTHrP(1-34), [Trp^1]PTHrP(1-36), PTH(2-38), PTH(3-34) and MPTH(1-14) stimulation of hPTH1R-mediated cAMP accumulation are shown in **Figure 1A** and EC_{50} values of 0.23, 0.39, 0.86, 3.6, 1.0, 241 and 0.33 nM were determined, respectively.

A collection of several N- and C-terminal truncated PTH and PTHrP peptide analogs with select AA substitutions was evaluated for agonist activity in the cAMP accumulation assay and **Table I** summarizes the results. All nontruncated peptide analogs, including PTH(1-37), PTHrP(1-37), PTHrP(1-34), [Tyr^1]PTH(1-34), [Nle^{8,18},Tyr^{34}]PTH(1-34), [Trp^1]PTHrP(1-36), and [Bpa^1]PTHrP(1-36), as well as PTH(2-38), were full agonists in promoting cAMP accumulation with potencies similar to PTH(1-34). The N-terminal truncated peptide analogs PTH(3-34) and [Nle^{8,18},Tyr^{34}]PTH(3-34)NH_2 displayed partial agonist activity and were 288- and 93-fold less potent than PTH(1-34), respectively, indicating that although removal or substitution of the first AA of PTH(1-34) or substitution of the first AA of PTHrP(1-34) does not alter

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agonist activity but removal of the first two AAs of PTH(1-34) markedly decreases agonist activity in agreement with previous studies (Gensure et al., 2005). Several additional PTH and PTHrP peptide analogs with deletion of the first seven N-terminal AAs, including [Nle^{8,18},Tyr^{34}]PTH(7-34)NH_2, [Tyr^{34}]PTH(7-34)NH_2, PTHrP(7-34)NH_2, PTH(7-34), [Asn^{10},Leu^{11},D-Trp^{12}]PTHrP(7-36)NH_2 and [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 were also found inactive in the cAMP accumulation assay. Furthermore, [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 completely antagonized stimulation of cAMP accumulation elicited by 0.8 nM PTH(1-34) with an IC_{50} value of 1 μM (**Figure 1B**). None of the inactive peptides reduced basal cAMP accumulation consistent with inverse agonist activity (data not shown).

The C-terminal truncated peptide analogs, including PTH(1-31), PTH(1-31)NH_2, MPTH(1-28) and [Gly^{1}Arg^{19}]PTH(1-28) stimulated cAMP accumulation with similar potency and efficacy as PTH(1-34) whereas PTHrP(1-16) was found inactive. Additional C-terminal truncated PTH peptide analogs containing AA substitutions designed to introduce steric hindrance and increase peptide α-helicity and side group interactions were also tested. ZP2307 (Ac_5c^1,Aib^3,Leu^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Asp^{17}]PTH(1-17)NH_2) (Neerup et al., 2011) and [Aib^{1,3},Nle^8, Gln^{10},Har^{11}]PTH(1-11)NH_2 (Caporale et al., 2010) were full agonists displaying 10- and 28-fold lower potencies than PTH(1-34) whereas the potency and efficacy of MPTH(1-14) (Okazaki et al., 2008) was similar to PTH(1-34).

PTH/PTHrP peptide analog stimulation of PTH1R-mediated G_q-signaling. Agonist activation of hPTH1R expressed in some cells leads to G_q-coupling, activation of phospholipase Cβ (PLCβ) and hydrolysis of membrane-associated phosphatidylinositol

species to form inositol phosphates, increases in intracellular calcium and activation of calcium dependent enzymes including PKC (Abou-Samra et al., 1992;Taylor and Tovey, 2012). PTH and PTHrP peptide analog stimulation of PTH1R-mediated IP_1 accumulation was measured as a parameter of activation of hPTH1R-mediated G_q-signaling and was accomplished using the CisBio IPone^{TM} TRF assay and the same CHO-K1 clone stably expressing hPTH1R used for cAMP assays. Representative concentration-response curves for stimulation of hPTH1R-mediated IP_1 accumulation by PTH(1-34), PTHrP(1-34), [Tyr^1]PTH(1-34), PTH(2-38), MPTH(1-28), ZP2307, and MPTH(1-14) are shown in **Figure 2** and EC_{50} values of 19, 27, >10,000, >10,000, 13, 9,014 and 1,212 nM were determined, respectively. The agonist activity of these and additional peptides in the hPTH1R IP_1 accumulation assay are summarized in **Table 1**.

The full length peptides including PTH(1-34), PTHrP(1-34), and [Nle^{8,18},Tyr^{34}]PTH(1-34) all posses similar agonist activity in the IP_1 accumulation assay (**Table 1**). However, peptides with N-terminal modification or truncation including [Bpa^1]PTHrP(1-36), [Trp^1]PTHrP(1-36), PTH(2-38), PTH(3-34), [Nle^{8,18},Tyr^{34}]PTH(3-34) and peptide analogs with the first seven AA residues deleted, were all found to be inactive. Regarding C-terminal modified peptides, both PTH(1-31) and [Cyclo(Glu^{22}-Lys^{26}),Leu^{27}]PTH(1-31)NH_2 displayed similar agonist potency as PTH(1-34) but peptide analogs with more extensive C-terminal truncation and AA substitutions, including PTH(1-28), [Gly^1,Arg^{19}]PTH(1-28), ZP2307 and MPTH(1-14), were weaker agonists in comparison to PTH(1-34) while [Aib^{1,3},Nle^8,Gln^{10},Har^{11}]PTH(1-11)NH_2 was inactive. Interestingly, MPTH(1-28) displayed similar agonist activity as PTH(1-34) in contrast to PTH(1-28) suggesting that introduction of N-terminal AA substitutions maximizing J-

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domain interaction enhance agonist activity. However, peptide analogs with more extensive C-terminal truncation and similar AA substitutions did not display enhanced G_q agonist activity. Collectively these results suggest that the first native N-terminal AA of both PTH and PTHrP are absolutely required for stimulating hPTH1R-mediated G_q agonist activity and that C-terminal truncation of PTH(1-34) past PTH(1-31) also results in a marked loss in agonist activity. In addition, the introduction of unnatural AAs into the N-terminus of PTH designed to presumably optimize interaction with the PTH1R J-domain increases agonist activity for only peptide analogs with moderate C-terminal truncation such as MPTH(1-28) but not for more extensively C-terminally truncated peptides including ZP2307, MPTH(1-14) and [Aib^{1,3},Nle^{8},Gln^{10},Har^{11}]PTH(1-11).

PTH/PTHrP peptide analog stimulation of PTH1R-mediated β-arrestin-2 recruitment. Agonist activation of hPTH1R not only stimulates G_s- and G_q-signaling, but also stimulates β-arrestin recruitment, β-arrestin-dependent signaling including activation of ERK1/2, β-arrestin-mediated hPTH1R internalization and desensitization (Bisello et al., 2002; Gesty-Palmer et al., 2006; Villardaga et al., 2010). Agonist stimulation of hPTH1R-mediated β-arrestin recruitment in CHO-K1 cells stably expressing recombinant hPTH1R was measured using the DiscoveRX PathFinder^{TM} assay kit based on the β-galactosidase enzyme complementation platform (Olson and Eglen, 2007; Bassoni et al., 2012). Representative concentration-response curves for stimulation of hPTH1R-mediated β-arrestin recruitment by PTH(1-34), [Tyr^{1}]PTH(1-34), PTHrP(1-34), [Bpa^{1}]PTHrP(1-36), [Trp^{1}]PTHrP(1-36), PTH(1-28), ZP2307 and MPTH(1-14) are shown in **Figure 3A** and EC_{50} values of 0.65, 33, 0.87, 11, 36, 44, 98 and 17 nM were obtained, respectively. [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 did not stimulate

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hPTH1R-mediated β-arrestin recruitment but completely antagonizes stimulation promoted by 15 nM PTH(1-31)NH_2 with an IC_{50} value of 275 nM (**Figure 3B**).

The time courses of hPTH1R-mediated β-arrestin recruitment elicited by maximally effective concentrations of PTH(1-34) (100 nM) and PTHrP(1-34) (100 nM) were evaluated to determine if the kinetics of β-arrestin recruitment might vary between different peptides (**Figure 4**). In addition, although initial testing of [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 up to a final assay concentration of 10 μM indicated that this PTH peptide analog was inactive in the β-arrestin recruitment assay (**Figure 3B**), it was further tested to determine if longer assay incubation periods may result in an agonist response. Both time courses of PTH(1-34) and PTHrP(1-34) stimulation of hPTH1R-mediated β–arrestin recruitment were similar and hyperbolic. In contrast, [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 was inactive during the duration of the three hour assay incubation period confirming this PTH peptide analog does not promote PTH1R-mediated β–arrestin recruitment in CHO-K1 cells expressing recombinant hPTH1R.

The same collection of PTH and PTHrP analogs tested in the cAMP accumulation assay was tested in the β-arrestin recruitment assay, and the results are summarized in **Table 1**. Full length peptides including PTH(1-37), PTHrP(1-37), PTHrP(1-34) and [$Nle^{8,18}$,Tyr^{34}]PTH(1-34) displayed similar potency and efficacy as PTH(1-34). The N-terminally modified peptides [Tyr^1]PTH(1-34), PTH(2-38), [Trp^1]PTHrP(1-36), and [Bpa^1]PTHrP(1-36) also promoted hPTH1R-mediated β-arrestin recruitment but were less potent (21-, 61-, 12, and 6-fold, respectively) and possess lower intrinsic activity than PTH(1-34) (56%, 15%, 42%, and 50%, respectively). Peptide analogs containing additional N-terminal truncation and AA substitutions, including PTH(3-34),

[Nle^{8,18},Try^{34}]PTH(3-34)NH_2, [Nle^{8,18},Tyr^{34}]PTH(7-34)NH_2, [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2, PTHrP(7-34) and PTH(7-34) did not promote an agonist response. Regarding C-terminally truncated and modified PTH peptide analogs, PTH(1-31), PTH(1-31)NH_2, MPTH(1-28) and [Cyclo(Glu^{22}, Lys^{26}),Leu^{27}]PTH(1-31)NH_2 were all full agonists with potencies similar to PTH(1-34). In contrast, PTH(1-28), [Gly^{1},Arg^{19}]PTH(1-28), ZP2307, MPTH(1-14) and [Aib^{1,3},Nle^{8},Gln^{10},Har^{11}]PTH(1-11)NH_2 were 33-, 58-, 82-, 16- and 451-fold less potent full agonists than PTH(1-34), respectively. However, MPTH(1-28) was found to be equipotent with PTH(1-34) indicating N-terminal AA substitutions increasing the α-helical structure of the N-terminal region counteract the negative effects of C-terminal truncation for PTH(1-28) but not for shorter peptide analogs. These results suggest that Ser^{1} and Ala^{1} of PTH and PTHrP, respectively, are important for potent full agonist activity in the hPTH1R β-arrestin recruitment assay and that C-terminal truncation past PTH(1-31) moderately decreases agonist potency to stimulate β-arrestin recruitment with the exception of MPTH(1-28). Furthermore, peptide modifications negatively impacting agonist activity in stimulating G_q-signaling have a similar effect on activity to promote β-arrestin recruitment without altering activity to stimulate hPTH1R-mediated G_s-signaling.

PTH/PTHrP peptide analog stimulation of ERK1/2 phosphorylation. Activation of hPTH1R expressed in osteoblasts and osteosarcoma cells leads to phosphorylation and activation of ERK1/2 by mechanisms that can involve both G-protein dependent signaling and G-protein independent and β-arrestin dependent signaling (Syme et al., 2005; Gesty-Palmer et al., 2006). PTH1R-mediated stimulation of ERK1/2 phosphorylation was measured using the CisBio TRF Cellul'Erk^{TM} assay kit and

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the same CHO-K1 cell stable clone expressing recombinant hPTH1R used for cAMP and IP_1 accumulation assays. Representative concentration-response curves for PTH(1-34), [Tyr^1]PTH(1-34), PTHrP(1-34), [Trp^1]PTHrP(1-36), MPTH(1-28), ZP2307 and MPTH(1-14) are shown in **Figure 5A** and EC_{50} values of 21, >10,000, 5, >10,000, 3, 151 and 114 nM were determined, respectively. [D-Trp^{12}, Tyr^{34}]PTH(7-34)NH_2 was inactive but antagonized stimulation of ERK1/2 phosphorylation promoted by 30 nM PTH(1-34) with an IC_{50} value of 38 nM (**Figure 5B**).

The time courses of PTH(1-34) and PTHrP(1-34) stimulation of hPTH1R-mediated ERK1/2 phosphorylation in CHO-K1 cells stably expressing hPTH1R consists of two temporal components, a rapid transient phase peaking at 10 minutes after peptide addition, and a slower sustained component remaining elevated above baseline 2 hours after peptide addition (**Figure 6)**. The amplitude of the initial phase of stimulation promoted by PTHrP(1-34) is approximately 60% of that of PTH(1-34) whereas the sustained component was similar. PTH(2-38) in the same experiment did not stimulate ERK1/2 phosphorylation during the 2 hour incubation period. In two additional time course studies, [Trp^1]PTHrP(1-36) promoted a small rapid increase in ERK1/2 activation without promoting sustained ERK1/2 activation. Although [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 is reported to stimulate ERK1/2 activation in HEK293 cells expressing recombinant hPTH1R (Gesty-Palmer et al., 2006), this peptide analog was inactive during a during a two hour incubation period (data not shown).

The possible contribution of PTH1R-mediated G_s/PKA and/or G_q/PKC signaling pathways to stimulation of ERK1/2 phosphorylation in CHO-K1 cells expressing recombinant hPTH1R was investigated using the PKA selective inhibitor H89 and the

PKC selective inhibitor chelerythrine chloride. Preincubation of CHO-K1 cells expressing hPTH1R with 10 μM H89 partially reduced the amplitude of the rapid temporal component of PTH1R-mediated ERK1/2 phosphorylation but did not appreciably alter the slower sustained component of phosphorylation (**Figure 7A**). Preincubation of CHO-K1 cells with 1 μM chelerythrine chloride did not effect either temporal component of PTH(1-34) stimulation of PTH1R-mediated ERK1/2 phosphorylation (**Figure 7B**). These results suggest that activation of PTH1R-mediated G_s-signaling leading to PKA activation contributes in part to the more transient rapid component of ERK1/2 activation whereas G_q-signaling leading to PKC activation does not contribute to either temporal component of activation.

Several of the PTH and PTHrP peptide analogs evaluated in hPTH1R cAMP, IP_1, and β-arrestin recruitment assays were also evaluated for ability to stimulate hPTH1R-mediated ERK1/2 phosphorylation and the results are summarized in **Table 1**. PTH(1-37), PTHrP(1-34), PTHrP(1-37), [$Nle^{8,18}$,Tyr^{34}]PTH(1-34), PTH(1-31), and [Cyclo(Glu^{22}-Lys^{26})Leu^{27}]PTH(1-31)NH_2 all displayed similar agonist potencies as PTH(1-34). Interestingly, PTHrP(1-34) and PTHrP(1-37) displayed partial agonist activity in stimulating hPTH1R-mediated ERK1/2 phosphorylation compared to PTH(1-34). [Tyr^1]PTH(1-34), [Bpa^1]PTHrP(1-36), [Trp^1]PTHrP(1-36), and PTH(2-38), all of which are found to display weak partial agonist activity in the β-arrestin recruitment assay, were found inactive in the ERK1/2 phosphorylation assay. Furthermore, all PTH and PTHrP peptide analogs with the deletion of two or more N-terminal AAs were also found to be inactive. Regarding C-terminally truncated and modified peptide analogs, MPTH(1-28) displayed similar agonist potency and efficacy as

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PTH(1-34) whereas PTH(1-28), [Gly^1,Arg^{19}]PTH(1-28), ZP2307, and MPTH(1-14) displayed lower potency and efficacy than PTH(1-34) with the exception that the statistical significance of the potentially lower efficacy of [Gly^1,Arg^{19}]PTH(1-28) could not be assessed because only two EC_{50} determinations were made. Finally, [$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11}]PTH(1-11)NH_2 was found to be inactive in stimulating hPTH1R-mediated ERK1/2 phosphorylation. These results indicate that both N- and C-terminal domains of PTH(1-34) and PTHrP(1-34) contribute to hPTH1R-mediated stimulation of ERK1/2 phosphorylation similar to stimulation of G_q-signaling and β-arrestin recruitment. Introduction of unnatural AAs into more extensively C-terminally truncated PTH peptides with the exception of MPTH(1-28), did not enhance agonist activity in the ERK1/2 phosphorylation assay.

PTH/PTHrP peptide analog stimulation of hPTH1R internalization. The capacity of PTH and PTHrP peptide analogs to promote hPTH1R internalization in U2OS cells stably expressing recombinant receptor was measured using the DiscoveRX PathHunter™ total (β-arrestin dependent and independent) internalization assay based on β-galactosidase enzyme complementation taking place when the larger EA portion of β-galactosidase constitutively expressed on the surface of endosomes interacts with PTH1R tagged with Prolink. Several cell backgrounds were evaluated during development of this assay including CHO-K1 cells but U2OS cells expressing recombinant hPTH1R consistently provided the largest agonist window (communication with DiscoveRX). Representative concentration-response curves for PTH(1-34), [Tyr^1]PTH(1-34), PTHrP(1-34), [Trp^1]PTHrP(1-36), PTH(1-28), ZP2307 and MPTH(1-14) are shown in **Figure 8** and EC_{50} values of 1, 50, 4, 120, 275, 1,423 and 239 nM were

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determined, respectively. The potency and efficacy of these and several other PTH and PTHrP peptide analogs are summarized in **Table I**.

PTHrP(1-34) and [Nle^{8,18},Tyr^{34}]PTH(1-34) were found to display similar potency and efficacy to promote receptor internalization as PTH(1-34). The N-terminal modified PTHrP peptide analogs [Trp^{1}]PTHrP(1-36) and [Tyr^{1}]PTH(1-34) stimulated hPTH1R internalization with lower potency (21- and 53-fold) and efficacy (48% and 75%) whereas the related peptide analog [Bpa^{1}]PTHrP(1-36) in addition to PTH(2-38) and PTH(3-34) were inactive. Regarding C-terminally modified peptides, PTH(1-31) and [Cyclo(Glu^{22}-Lys^{26})Leu^{27}]PTH(1-31)NH_2 were found to have similar agonist activity as PTH(1-34) whereas PTH(1-28), [Gly^{1},Arg^{19}]PTH(1-28), ZP2307, and MPTH(1-14) were less potent (53-, 136-, 342-, and 47-fold lower, respectively) than PTH(1-34) and [Aib^{1,3},Nle^{8},Gln^{10},Har^{11}]PTH(1-11) was inactive. Interestingly, PTH(1-28), [Gly^{1},Arg^{19}]PTH(1-28), ZP2307 and MPTH(1-14) all stimulated significantly higher levels of hPTH1R internalization than PTH(1-34). Conversely, MPTH(1-28) displayed similar potent agonist activity as PTH(1-34) but was significantly less efficacious. These results suggest that substitution of the first AA of PTH or PTHrP or removal of the first AA of PTH results in a complete loss of ability to promote internalization. In addition, additional C-terminal truncation of PTH from PTH(1-31) and introduction of AA substitutions optimizing J-domain interaction with the exception of MPTH(1-28) results in a decrease in agonist potency while increasing the capacity to promote hPTH1R internalization.

Comparison of potencies of PTH/PTHrP peptide analogs to stimulate hPTH1R-mediated signaling pathways. To facilitate comparison of the agonist potency of PTH/

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PTHrP peptides in the different hPTH1R functional assays, EC_{50} values were converted to pEC_{50} values and plotted for each peptide analog (**Figure 9)**. Regarding full length peptides, the observed agonist potency rank order for PTH(1-34), PTHrP(1-34) and [$Nle^{8,18}$,Tyr^{34}]PTH(1-34) was cAMP accumulation = β-arrestin recruitment = internalization > ERK1/2 phosphorylation = IP_1 accumulation (**Figure 9A**). The potency rank order of N-terminally modified peptides [Tyr^1]PTH(1-34) and [Trp^1]PTHrP(1-36) was cAMP accumulaltion = β-arrestin > internalization and no agonist activity in the IP_1 accumulation and ERK1/2 phosphorylation assays. [Bpa^1]PTHrP(1-36) displayed a similar agonist profile as [Trp^1]PTHrP(1-36) and [Tyr^1]PTH(1-34) with the exception that it does not promote hPTHR1 internalization.

Agonist potency profiles determined for N-terminally truncated PTH peptide analogs are shown in **Figure 9B**. As previously discussed, removal of the Ser^1 of PTH does not impact agonist activity in the cAMP assay, reduces agonist potency and efficacy to promote β-arrestin recruitment and results in a complete loss of agonist activity in IP_1 accumulation, ERK1/2 phosphorylation, and receptor internalization assays. Furthermore, removal Ser^1 and Val^2 of PTH(1-34) ((PTH(3-34) and [$Nle^{8,18}$,Tyr^{34}]PTH(3-34)) also results in a significant reduction in agonist potency and efficacy to stimulate G_s-signaling. **Figure 9B** clearly shows that N-terminal truncation of PTH and PTHrP has a greater negative impact on stimulation of hPTH1R-mediated G_q-signaling, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization than on stimulation of G_s-signaling. Furthermore, all PTH/PTHrP analogs with deletion of seven N-terminal amino acids were found inactive all five hPTH1R functional assays which is consistent

with the proposed requirement of these AAs for interaction with the hPTH1R J-domain (**see Table I**).

The agonist potency profiles for C-terminally truncated and modified PTH and PTHrP peptide analogs tested in all five hPTH1R functional assays are shown in **Figure 9C**. Removal of the last three C-terminal AAs of PTH(1-34) does not greatly alter agonist activity in any of the five hPTH1R functional assays compared to PTH(1-34). PTH peptides with further truncation and AA substitutions including PTH(1-28), [Gly^{1},Arg^{19}]PTH(1-28), ZP2307, and MPTH(1-14) generally have the potency rank order of cAMP > β–arrestin = ERK1/2 > receptor internalization >IP_1 accumulation whereas MPTH(1-28) possesses a similar assay potency rank order as PTH(1-34). Finally, while [$Aib^{1,3}$,Nle^{8},Gln^{10},Har^{11}]PTH(1-11)NH_2 has only a modest decrease in agonist potency in the cAMP assay, a more significant decrease in potency to stimulate β-arrestin recruitment, and no agonist activity in IP_1 accumulation, ERK1/2 phosphorylation and receptor internalization assays were observed. These results suggest that PTH(1-34) peptide analogs with C-terminal truncation of six AAs and substitution of hydrophobic AAs with unnatural AAs increasing N-terminal α-helicity and side chain interactions (e.g. MPTH(1-28)) helps to maintain agonist potency to promote G_q-signaling, β-arrestin recruitment and ERK1/2 phosphorylation (e.g. MPTH(1-28)). However, agonist activity of more extensively truncated peptide analogs with these modifications is not maintained. Importantly, these results indicate that C-terminal AAs of PTH(1-34) generally considered critical in primarily mediating binding to the PTH1R N-terminus also contribute to peptide docking into the J-domain and subsequent stimulation of both G-protein dependent and G protein independent PTH1R-mediated signaling.

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Biased PTH/PTHrP peptide analogs. The correlation of the different PTH and PTHrP peptide analogs to stimulate hPTH1R-mediated cAMP accumulation, IP_1 accumulation, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization were also determined for peptides found active in all five hPTH1R functional assays (**Figure 10**). This was accomplished by comparing calculated E_{Max}/EC_{50} values for PTH and PTHrP peptide analogs within each assay pairing and performing a Deming linear regression analysis of the data. Several of the PTH and PTHrP peptide analogs evaluated in this study were found to stimulate hPTH1R-mediated G_s-signaling but were devoid of agonist activity in other functional assays consistent with biased agonist activity. Additional peptide analogs were also found to display more subtle differences in efficacy and/or potency in cAMP accumulation, IP_1 accumulation, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization assays and the "equiactive comparison" method recently described by Rajagopal et al., (2011) was used to quantify biased agonism for these peptides. Using this approach, concentrations of peptide analog and PTH(1-34) required for an equiactive response for one signaling pathway vs. another are extrapolated from fits of each concentration-effect curves and a bias factor, referred to as β (equation described in Materials and Methods), is calculated. The bias factor represents the relative ability of a given PTH/PTHrP peptide analog to stabilize one hPTH1R-mediated signaling pathway over another on a logarithmic scale compared with PTH(1-34) (Rajagopal et al., 2011).

PTH/PTHrP peptide analogs displaying statistically significant ($P < 0.05$) bias compared to PTH(1-34) for stimulating hPTH1R-mediated G_s-signaling vs. G_q-signaling compared to PTH(1-34) include the C-terminally modified peptides [Gly^1,Arg^{19}]PTH(1-

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28) and MPTH(1-14) (**Figure 11A**). PTH and PTHrP peptide analogs promoting significant biased hPTH1R-mediated G_s-signaling over β-arrestin-recruitment include PTH(2-38), [Tyr^1]PTH(1-34), [Gly^1,Arg^{19}]PTH(1-28), MPTH(1-14) and [Aib^{1,3},Nle^8,Gln^{10},Har^{11}]PTH(1-11)NH_2 (**Figure 11B**). None of the peptide analogs evaluated displayed agonist bias for stimulation of hPTH1R-mediated G_s-signaling vs. ERK phosphorylation (data not shown) but several peptide analogs including [Gly^1,Arg^{19}]PTH(1-28), [Tyr^1]PTH(1-34), MPTH(1-14), ZP2307, [Trp^1]PTHrP(1-36) and PTH(1-28) were found to promote significant biased G_s-signaling over receptor internalization compared to PTH(1-34) (**Figure 11.C**) while [Cyclo(Glu^{22}-Lys^{26})Leu^{27}]PTH(1-31)NH_2 and PTHrP(1-34) display a slight bias for activating receptor internalization over G_s-signaling. No significant signaling bias was observed for any PTH/PTHrP peptide analogs evaluated for simulation of PTH1R-mediated G_q-signaling vs. β-arrestin recruitment, G_q-signaling vs. ERK1/2 phosphorylation, G_q-signaling vs. internalization, β-arrestin recruitment vs. ERK1/2 phosphorylation, β-arrestin recruitment vs. receptor internalization or ERK1/2 phosphorylation vs. receptor internalization (data not shown) compared to PTH(1-34).

DISCUSSION

Studies of N- and C-terminally modified PTH/PTHrP peptide analogs in PTH1R radioligand binding and functional assays capturing G_s- and G_q-signaling suggest the N-terminal domain (AA 1-14) is important for stimulating PTH1R-mediated G protein signaling, and the C-terminal domain (AA23-34) contributes to receptor binding and PLCβ-independent PKC activation (Gensure et al., 2005; Villardaga et al., 2010). Peptide domains contributing to stimulation of hPTH1R-mediated β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization are not well defined. Therefore, several PTH/PTHrP peptide analogs were evaluated in orthogonal functional assays capturing not only PTH1R-mediated G_s- and G_q-signaling, but also β-arrestin recruitment, ERK1/2 activation and receptor internalization to provide a more comprehensive understanding of regions contributing to hPTH1R signaling and further define patterns of biased agonist activity. All hPTH1R assays except receptor internalization were performed using a CHO-K1 clone stably expressing hPTH1R and another engineered CHO-K1 clone expressing prolink-tagged hPTH1R and β-arrestin-EA was used for the β-arrestin recruitment assay. It is possible levels of hPTH1R expression in these two CHO-K1 clones as well as U2OS cells employed for the internalization assay may differ which could impact agonist efficacy. Similar to a study using HEK293 cells expressing hPTH1R (Gesty-Palmer et al., 2006), stimulation of hPTH1R-mediated ERK1/2 phosphorylation in CHO-K1 cells consists of two temporal components consisting of rapid transient G-dependent and slower sustained β-arrestin-dependent phases. Strong correlations between PTH/PTHrP peptide EC_{50} values for stimulation of G_s/G_q- signaling

and β-arrestin recruitment and β-arrestin recruitment and receptor internalization were noted implying they are likely interdependent signaling pathways.

Structural domains involved in stimulation of different PTH1R-mediated signaling pathways. PTH(1-34) and PTHrP(1-34) display similar agonist activities in all hPTH1R CHO-K1functional assays with a potency rank order of cAMP = β-arrestin recruitment = internalization > ERK1/2 phosphorylation > IP_1. Higher concentrations of PTH(1-34) are reportedly required for stimulation of hPTH1R-mediated G_q- than G_s-signaling (Taylor and Tovey, 2012) which was also observed in this study. The significance of the observation that PTHrP(1-34) and PTHrP(1-37) are partial agonists in the ERK1/2 phosphorylation assay is unclear. PTHrP(1-34) was found to promote similar levels of receptor internalization as PTH(1-34).

Evaluation of N-terminally modified peptides including [Trp^1]PTHrP(1-36), [Tyr^1]PTH(1-34) and PTH(2-34) indicates the first native AA of either PTH and PTHrP is not required for stimulation of G_s agonist activity but plays a critical role in facilitating stimulation of G_q, β-arrestin recruitment, ERK1/2 phosphorylation and internalization. In contrast, Val^2 of PTH is required for agonist activity in all hPTH1R functional assays as previously reported for G_s- and G_q-signaling (Takasu et al., 1999). The inability of [Bpa^1]PTHrP(1-36) to promote hPTH1R internalization has been reported by others (Bisello et al., 2002). Results of this study do not support previous claims that [Trp^1]PTHrP(1-36) and [Bpa^1]PTHrP(1-36) are solely G_s-biased agonists (Bisello et al., 2002, Gesty-Palmer et al., 2006) and that [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 is a β-arrestin biased agonist capable of stimulating ERK1/2 phosphorylation (Gesty-Palmer et al., 2006; 2009). Furthermore, PTH(7-34)NH_2 does not promote receptor internalization in

CHO-K1 cells as reported for DTC cells expressing hPTH1R (Sneddon et al., 2004). The differences between the results these studies may be due to a combination of expression of hPTH1R in different cell backgrounds (e.g. CHO-K1 vs HEK293), different levels of receptor expression, or use of different assay methods. For example, β-arrestin recruitment has been previously measured by translocation of GFP-tagged arrestin (Bisello et al., 2002; Gesty-Palmer et al., 2006) whereas the β-arrestin recruitment assay directly measures binding of β-arrestin to agonist activated receptor, is not impacted by receptor reserve and agonist EC_{50} values correlate well with receptor affinity enabling a clear separation of affinity and efficacy of GPCR agonists (Nickolls et al., 2011).

Evaluation of the agonist activity of C-terminally modified PTH peptides indicate removal of the last six AA residues of PTH(1-34) does not effect ability to stimulate G_s-signaling but does decrease agonist activity in all the other hPTH1R assays. Furthermore, the efficacy of peptides with truncation past PTH(1-31) to stimulate hPTH1R-mediated ERK phosphorylation and receptor internalization is decreased and increased, respectively. In contrast, the agonist profile of MPTH(1-28) is similar to PTH(1-34) suggesting that N-terminal AA substitutions increasing α-helicity of the N-terminal domain (Okazaki et al., 2007) help maintain agonist activity of PTH(1-28) analogs. However, introduction of the same AA substitutions into more extensive C-terminal truncated peptides helps predominately to maintain agonist activity to stimulate G_s-signaling as reported by others (Shimizu et al., 2001; 2004; Caporale et al., 2009; 2010; Neerup et al., 2011). The structural basis for the greater capacity of PTH(1-28), [Gly^1,Arg^{19}]PTH(1-28), ZP2307 and MPTH(1-14) to stimulate receptor internalization is

unclear but may be a result of C-terminal AAs present in PTH(1-31) providing a restraint on receptor internalization.

The structural basis for the differences in the negative impact of PTH/PTHrP peptide N- and C-terminal modifications on stimulation of PTH1R-mediated G_s-signaling compared to other signaling pathways is unclear. However, our experimental results are consistent with previous reports that the first two AAs play an important role in mediating peptide activation of the J-domain and G_s-signaling (Gensure et al., 2005) in addition to activation of other hPTH1R signaling pathways evaluated. Although the high resolution X-ray crystal structure of PTH(15-34) and PTHrP(12-34) bound to the extracellular domain (ECD) of the hPTH1R receptor have been reported (Pioszak et al., 2008; 2009), structural details regarding the interaction between the peptide N-terminal and receptor J-domains are not available. Although the two-site model of PTH1R activation posits that the C-terminal domain of PTH (AA 23-34) predominately mediates binding of peptide to the extracellular N-terminus (Vilardaga et al., 2010), our results also suggest that the C-terminal domain also plays an important role in activation of all hPTH1R signaling pathways as well as receptor internalization. Vilaradaga et al. (2010) have suggested that the two-site model may be an oversimplification and hypothesize that bound peptide may adopt a folded, rather than an extended conformation upon binding to receptor, and that the ligand-binding surface of receptor N- and J-domains are in close proximity to each other in the agonist bound state. Additional structural studies of PTH(1-34) and PTHrP(1-34) bound to full length hPTH1R will help to further elucidate the roles agonist peptide N- and C-terminal domains play in activating the J-domain.

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Patterns of hPTH1R-mediated signaling by PTH and PTHrP peptide analogs.

Previous *in vivo* studies using reported biased PTH/PTHrP peptide analogs suggest that PTH1R-mediated G_s-signaling plays a predominant role in anabolic actions of intermittent PTH(1-34) (Rixon et al., 1995; et al., 1996; Yang et al., 2007; Rhee et al., 2006) whereas G_q-signaling does not. Furthermore, [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2, a proposed β-arrestin biased agonist, is reported to promote bone formation that is lost with ablation of β-arrestin (Gesty-Palmer et al., 2009). However, we observe that patterns of hPTH1R mediated signaling promoted by some of these peptide analogs are more complex or different from previous reports. During revision of this manuscript, van der Lee et al. (2012) report that [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 does not stimulate β-arrestin recruitment or ERK1/2 phoshorylation in CHO-K1 cells expressing recombinant hPTH1R but does antagonize agonist response similar to results of this study (van der Lee et al., 2012). Thus, inconsistencies regarding therapeutically relevant hPTH1R signaling required for bone formation exist and further studies using PTH/PTHrP peptide analogs with well-defined signaling patterns are required to further address this important question.

Several PTH/PTHrP peptide analogs evaluated in this study promote differential PTH1R-mediated signaling consistent with biased agonism however the contribution of differences in strength of signaling cannot be ruled out. The use of the same CHO-K1 cells for all functional assays with the exception of internalization lessens the possibility that differences are solely due to the latter phenomenon. All of the peptides promoting differential signaling activated G_s-signaling and none displayed a reversal in potency or efficacy rank order in the other PTH1R functional assays which is considered a hallmark

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of biased GPCR signaling but is not a requirement (Kenakin 2004, 2007; Urban et al., 2007). One question arising from this study is whether biased PTH/PTHrP peptide analogs can be designed that exclusively activate other signaling pathways but not G_s-signaling.

A prerequisite to additional efficacy studies using well-defined signaling biased PTH/PTHrP peptide analogs to ascertain therapeutically relevant hPTH1R signaling pathways will be to confirm biased signaling patterns of peptide analogs using therapeutically important osteoblasts. Additionally, *in vitro* efficacy studies of the effect of different biased peptides on osteoblast proliferation, differentiation, apoptosis and function could then be used to guide which biased PTH/PTHrP peptides should be prioritized for *in vivo* studies of rodent bone formation. Potentially useful peptide analogs for this purpose could include [Trp^1]PTHrP, [Tyr^1]PTH(1-34), [Bpa^1]PTHrP(1-36), MPTH(1-14) and [$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11}]PTH(1-11). In addition, we have recently discovered a PTH analog with potent G_s-biased agonist activity that would also be useful for these studies (Gineste et al. manuscript in preparation). Once therapeutically desirable PTH1R-mediated signaling is further established in *in vivo* models of osteoporosis, peptides promoting restricted desirable increases in bone formation without resorption could potentially be designed to provide a safer and more effective treatment of osteoporosis. Indeed, discovery of biased GPCR agonists with superior therapeutic profiles has recently created a valuable opportunity for the pharmaceutical industry (Reiter et al., 2012).

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FIGURES/LEGENDS

Figure 1. (A) Representative concentration-response curves for PTH/PTHrP peptide analog stimulation of hPTH1R-mediated cAMP accumulation. Representative concentration-response curves for PTH(1-34) (solid circles), PTHrP(1-34) (solid squares), [Tyr^1]PTH(1-34) (solid triangles), [Trp^1]PTHrP(1-36) (solid inverted triangles), PTH(2-38) (solid diamonds), PTH(3-34) (open circles), PTH(1-28) (open squares), ZP2307 (open triangles) and MPTH(1-14) (open inverted triangles) stimulation of hPTH1R-mediated cAMP accumulation in CHO-K1 cells stably expressing recombinant hPTH1R are shown. Assay incubations were performed for 30 min at 37°C. Each data point represents the mean+/-SEM of % control values based on the maximal signal obtained with PTH(1-34) derived from duplicate or triplicate determinations made at each indicated test peptide concentration. Agonist EC_{50} values were determined as described in Materials and Methods. **(B) [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 antagonism of PTH(1-34) stimulation of cAMP accumulation**. CHO-K1 cells expressing recombinant hPTH1R were preincubated with indicated concentrations of [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 for 15min at 37°C prior to addition of 0.8nM PTH(1-34), followed by an additional 30 min incubation at 37°C. Each data point represents the mean+/-SEM ALU from duplicate determinations at each indicated [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 concentration. The IC_{50} value was determined as described in Materials and Methods. The typical basal signal observed was 8,309+/-117 ALU and the signal obtained in the presence of 10 μM [D-Trp^{12},Tyr^{34}]PTH(7-34)NH2 was 8,184+/-98 ALU indicating full inhibition of agonist stimulation.

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Figure 2. Representative concentration-effect curves for PTH/PTHrP peptide analog stimulation of hPTH1R-mediated increases in IP_1 accumulation. Representative concentration effect curves for PTH(1-34) (solid circles), PTHrP(1-34) (solid squares), [Tyr^1]PTH(1-34) (solid triangles), PTHrP(1-36) (solid triangles), PTH(2-38) (solid inverted triangles), MPTH(1-28) (open circles), ZP2307 (open squares), and MPTH(1-14) (open triangles) stimulation of hPTH1R-mediated IP_1 accumulation in CHO-K1 cells stably expressing recombinant receptor are shown. Assay incubations were conducted for 90 min at 37°C. Each data point represents the mean+/-SEM of % control values based on the maximal signal obtained with PTH(1-34) and were derived from duplicate or triplicate determinations made at each indicated test peptide concentration. Agonist EC_{50} values were determined as described in Materials and Methods.

Figure 3. (**A**) **Representative concentration response curves for PTH/PTHrP peptide analog stimulation of hPTH1R-mediated β-arrestin recruitment.** Representative concentration-response curves for PTH(1-34) (solid circles), PTHrP(1-34) (solid squares), [Tyr^1]PTH(1-34) (solid triangles), [Bpa^1]PTHrP(1-36) (inverted solid triangles), [Trp^1]PTHrP(1-36) (open circles), PTH(1-28) (open squares), ZP2307 (open triangles) and MPTH(1-14) (open inverted diamonds) for stimulation of β-arrestin recruitment in CHO-K1 cells stably expressing recombinant hPTH1R are shown. Assay incubations were performed for 90 min at 37°C. Each data point represents the mean+/-SEM of % control values based on ALU obtained in the presence of a maximal concentration of PTH(1-34) and were derived from duplicate or triplicate determinations made at each

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indicated test peptide concentration. EC_{50} values were determined as described in Materials and Methods. (**B**) **[D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 antagonism of PTH(1-31) stimulation of hPTH1R-mediated β-arrestin recruitment.** Concentration-dependent inhibition of PTH(1-31) mediated stimulation of β-arrestin recruitment by [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 in CHO-K1 cells stably expressing hPTH1R is shown. Cells were preincubated for 15 min at 37^{o}C with indicated concentrations of [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 followed by addition of 15nM PTH(1-31) and an additional 90 min incubation at 37^{o}C. Each data point represents the mean+/-SEM of ALU derived from duplicate determinations at each indicated concentration of [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 and the IC_{50} value was determined as described in Materials and Methods. The basal assay signal obtained in the absence of added PTH(1-31) was 9207+/-276 ALU and the signal and in the presence of 1 μM [D-Trp^{12},Tyr^{34}]PTH(7-34) was 44,830+/-1685 ALU corresponding to 8% of the signal obtained in the presence of 15 nM PTH(1-31) alone.

Figure 4. Time courses of PTH(1-34) (100 nM) (closed circles), PTHrP(1-34) (100 nM) (solid squares) and [D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 (10 μM) (solid triangles) stimulation of PTH1R-mediated β-arrestin recruitment in CHO-K1 cells stably expressing recombinant hPTH1R are shown. Each data point represents the mean+/-SEM of ALU obtained from duplicate determinations at each indicated time point. Data for PTH(1-34) and PTHrP(1-34) were fit to a simple hyperbolic function as described in Materials and Methods.

Figure 5. (**A**) **Representative concentration-response curves for PTH and PTHrP peptide analog stimulation of hPTH1R mediated phosphorylation of ERK1/2.** Representative concentration-effect curves for PTH(1-34) (solid circles), PTHrP(1-34) (solid squares), [Tyr^1]PTH(1-34) (solid triangles), [Trp^1]PTHrP(1-36) (solid inverted triangles), MPTH(1-28) (open circles), ZP2307 (open squares) and MPTH(1-14) (open triangles) stimulation of hPTH1R-mediated ERK1/2 phosphorylation in CHO-K1 cells stably expressing hPTH1R are shown. Assay incubations were conducted for 15 min at room temperature. Each data point represents the mean+/-SEM of the fold stimulation of ERK1/2 phosphorylation over basal phosphorylation and duplicate determinations were made at each indicated test peptide concentration. EC_{50} values were determined as described in Materials and Methods. (**B**) [**D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2 antagonism of PTH(1-34) mediated stimulation of ERK1/2 phosphorylation**. CHO-K1 cells expressing recombinant hPTH1R were preincubated for 15 min at room temperature with antagonist prior to addition of 30 nM PTH(1-34) and an additional incubation of 15 min at room temperature. Each data point represents the mean+/-SEM of ALU obtained from duplicate determinations made at each indicated concentration of [D-Trp^{12},Tyr^{34}] PTH(7-34)NH_2 and the IC_{50} was determined as described in Materials and Methods. The basal AFU obtained in the absence of PTH(1-34) was 2744+/-212 and the AFU obtained in the presence of 1 μM [D-Trp^{12},Tyr^{34}]PTH(7-34)NH2 was 3086+/-43 indicating full inhibition of the agonist response.

Figure 6. Time courses of PTH(1-34), PTHrP(1-34), and [D-Trp^{12},Try^{34}]PTH(7-34)NH_2 stimulation of hPTH1R-mediated ERK1/2 phosphorylation. The time courses

of stimulation of ERK1/2 phosphorylation in the presence of no added peptide (solid circles), 1 μM PTH(1-34) (solid squares), 1 μM PTHrP(1-34) (solid inverted triangles), and 10 μM PTH(2-38) (closed triangles) in CHO-K1 cells stably expressing hPTH1R are shown. Assay incubations were conducted at 37^{o}C for the indicated incubation intervals. Data points represent the mean+/-SEM of the ratio fluorescence readings taken at 665/625 nm from three separate determinations made at each time point.

Figure 7. **Influence of PKA and PKC inhibitors on the time course of PTH(1-34) stimulation of ERK1/2 phosporylation.** The effects of 10 μM H89 (A) and 10 μM chelerythrine (B) on the time course of stimulation of ERK1/2 phosphorylation mediated by 1 μM PTH(1-34) in CHO-K1 cells expressing recombinant hPTH1R are shown. Cells were either preincubated for 15 min at room temperature without (solid circles and triangles) or with kinase inhibitor (solid squares) prior to no addition (solid circles) or addition of 1 μM PTH(1-34) (solid squares and triangles) and time course evaluation of ERK1/2 phosphorylation. Data points represent the mean+/-SEM of the ratio of fluorescence readings taken at 665/625 nm from three separate determinations made at each time point.

Figure 8. Representative concentration-effect curves for PTH/PTHrP peptide analog stimulation of hPTH1R internalization. Representative concentration-effect curves for PTH(1-34) (solid circles), PTHrP(1-34) (solid squares), [Tyr^{1}]PTH(1-34) (solid triangles), [Trp^{1}]PTHrP(1-36) (solid inverted triangles), PTH(1-28) (open circles), ZP2307 (open squares) and MPTH(1-14) (open triangles) stimulation of hPTH1R

internalization in U2OS cells stably expressing hPTH1R are shown. Incubations were conducted for a period of 3 hr at 37^{o}C. Each data point represents the mean+/-SEM of % control values based on maximal receptor internalization elicited by PTH(1-34) and were derived from duplicate determinations at each indicated test peptide concentration. EC_{50} values were determined as described in Materials and Methods.

Figure 9. Comparison of potencies of PTH/PTHrP peptide analogs to stimulate different hPTH1R-mediated cAMP accumulation, IP_1 accumulation, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization. (A) Comparison of agonist potencies for full-length peptides, (B) N-terminal modified and (C) C-terminal modified PTH and PTHrP peptides are shown. EC_{50} values were converted to pEC_{50} values (negative logarithm of the EC_{50} value) and each data point represents the mean +/- SEM of the number of independent determinations listed in **Table I.**

Figure 10. Relation of PTH and PTHrP peptide analog agonist activities in stimulating cAMP accumulation IP_1 accumulation, β-arrestin recruitment, ERK1/2 phosphorylation and receptor internalization. Agonist activity of PTH and PTHrP peptide analogs was calculated by dividing E_{max} by EC_{50} values for each peptide taken from Table I, values were plotted for each assay pairing, and the data were subjected to Deming (Mode II) linear regression analysis using Prism.

Figure 11. Bias factors obtained for different PTH/PTHrP peptides. hPTH1R signaling bias factors determined by the equiactive comparison method (see Materials

and Methods) are shown for G_s- vs. G_q-signaling (A), G_s-signaling vs. β-arrestin recruitment (B) and G_s-signaling vs. receptor internalization (C). Peptides displaying significant bias ($P < 0.05$, one-way Anova with a Tukey's post test) are shown with an asterisk.

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Table I. Potency and efficacy of PTH/PTHrP peptides for stimulation of cAMP accumulation, β-arrestin recruitment, IP_1 accumulation, ERK1/2 phosphorylation, and receptor internalization. EC_{50} values represent the mean+/-SEM of the indicated number of independent determinations in which 8-10 different assay concentrations were tested in either duplicate or triplicate. Agonist efficacy is based on the maximal response of PTH(1-34) obtained in the same assay plate. EC_{50} values were determined as described in Materials and Methods.

Peptide	***cAMP EC_{50} (nM)*** ***Mean+/-SEM (n)*** ***Efficacy (% PTH)***	***IP_1 EC_{50} (nM)*** ***Mean+/-SEM (n)*** ***Efficacy (% PTH)***	***β-Arrestin EC_{50} (nM)*** ***Mean+/-SEM (n)*** ***Efficacy (% PTH)***	***ERK1/2 EC_{50} (nM)*** ***Mean+/-SEM (n)*** ***Efficacy (% PTH)***	***Internalization*** ***Mean+/-SEM (n)*** ***Efficacy (% PTH)***
Full Length PTH/PTHrP Peptide Analogs					
PTH(1-34)	0.6+/-0.1 (52)[b,d] 100	30+/-4 (8)[c,e] 100	1.3+/-0.1 (36)[d] 100	16+/-3 (18) 100	5.0+/-0.8 (12)[d] 100
PTHrP(1-34)	2.0+/-0.7 (3)*[b] 100+/-4[d]	51+/-18 (3)[e] 103+/-7[d,e]	1.9+/-1.2 (3) 95+/-5	19+/-8 (5) 83+/-4**	3.0+/-0.5 (3) 110+/-8**
PTH(1-37)	0.6+/-0.1 (3) 105+/-3	ND	4.7+/-1.4 (4)* 93+/-5	13+/-6 (3) 111+/-13	ND
PTHrP(1-37)	0.4+/-0.1 (4)[d] 103+/-3[d]	ND	0.9+/-0.2 (4)[d] 100+/-1[d]	10+/-3 (4) 74+/-3**[a,c]	ND
[Bpa^1]PTHrP(1-36)	1.0+/-0.1 (3)[c] 102+/-3[c]	>10,000 (2)	7.3+/-1.0 (3) 50+/-6**	>10,000 (2)	>10,000 (3)
[Trp^1]PTHrP(1-36)	2.1+/-0.7(3)*[e] 110+/-4[c,e]	>10,000 (2)	15+/-9 (3)*[e] 42+/-5**	>10,000 (2)	103+/-15 (3)* 48+/-10**
[Tyr^1]PTH(1-34)	0.7+/-0.3 (4)[c,e] 100+/-6[c,e]	>10,000 (2)	27+/-4 (3)*[e] 56+/-7**	>10,000 (2)	104+/-29 (3)* 75+/-4**
[$Nle^{8,18}$,Tyr^{34}]PTH(1-34)	0.4+/-0.1 (3)[b] 99+/-1	56+/-38 (2)[e] 87+/-9	3.4+/-0.7 (3)[b] 93+/-3	18+/-2 (3) 68+/-11	5.4+/0.5 (2) 107+/-2
N-Terminal Truncated PTH/PTHrP Peptide Analogs					
PTH(2-38)	0.7+/-0.1 (3)[c]	>10,000 (2)	79+/-19 (4)*	>10,000 (4)	>10,000 (2)
PTH(2-38)	0.7+/-0.1 (3)[c] 99+/-2[c]	>10,000 (2)	79+/-19 (4)* 12+/-0.6**	>10,000 (4)	>10,000 (2)

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PTH(3-34)	173+/-38 (4) * 53+/-7**	>10,000 (2)	>10,000 (3)	>10,000 (2)	>10,000 (2)
[Nle^{8,18},Tyr^{34}]PTH(3-34)NH_2	56+/-10 (3)* 43+/-3**	ND	>10,000 (3)	>10,000 (2)	ND
[D-Trp^{12},Tyr^{34}]PTH(7-34)NH_2	>10,000 (2)	ND	>10,000 (3)	>10,000 (5)	>10,000 (2)
[Nle^{8,18},Tyr^{34}]PTH(7-34)	>10,000 (2)	ND	>10,000 (2)	>10,000 (2)	ND
[Tyr^{34}]PTH(7-34)NH_2	>10,000 (2)	ND	>10,000 (2)	>10,000 (2)	ND
PTHrP(7-34)NH_2	>10,000 (2)	ND	>10,000 (3)	>10,000 (2)	>10,000 (2)
PTH(7-34)	ND	ND	>10,000 (1)	>10,000 (2)	>10,000 (1)
[Asn^{10},Leu^{11},D-Trp^{12}]PTHrP(7-34)NH_2	>10,000 (2)	ND	>10,000 (2)	>10,000 (2)	ND
C-Terminal Truncated PTH/PTHrP Peptide Analogs					
PTH(1-31)	1.3+/-0.6 (3) 108+/-8	33+/-10 (2) 88+/-2	3.6+/-0.5 (3) 91+/-1**	35+/-5 (4) 85+/-16	6.4+/-1.9 (2) 100+/-2
PTH(1-31)NH_2	0.6+/-0.3 (4)^{d} 96+/-10	ND	4.7+/-0.6 (4)*^{d} 93+/-5	9.55+/-0.35 (2) 123+/-8	ND
(Cyclo(Glu^{22}-Lys^{26}),Leu^{27}]PTH(1-31)NH_2	0.8+/-0.1 (3) 105+/-2	23+/-13 (2) 100+/-3	0.8+/-0.2 (3) 110+/-7	11+/-3 (2) 96+/-11	0.51+/-0.03 (2) 75+/-11
PTH(1-28)	2.9+/-0.7 (5)*^{b} 105+/-3^{d}	1529+/-375 (2)*^{d,e**} 99+/-3	43+/-1 (2)*^{b} 94+/-18	98+/-14 (3)* 51+/-13**^{e}	268+/-45(4)* 140+/-6**
MPTH(1-28)	0.4+/-0.2 (2)^{b,d} 101+/-12^{e}	10+/-1 (2)^{de} 97+/-3^{e}	0.4+/-0.1 (2)^{b,d} 108+/-8^{e}	7+/-3 (2) 110+/-2^{e}	1.1+/-0.3 (3) 63+/-6**
[Gly^{1}Arg^{19}]PTH(1-28)	0.9+/-0.2 (2)^{b,e} 98+/-2	2692+/-99 (2)*^{d} 106+/-8	77+/-5 (2)*^{b} 82+/-12	53+/-31 (2)^{e} 49+/-18^{e}	680+/-148 (3)* 140+/-14**
ZP2307	6.2+/-1.2 (5)*^{b} 113+/-3*	5,327+/-2044 (2)*^{d} 85+/-1	107+/-4 (3)*^{b} 91+/-6	213+/-69* (3) 53+/-12**	1,714+/-185 (3)* 152+/-4**

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PTHrP(1-16)	>10,000 (1)	>10,000 (1)	>10,000 (2)	>10,000 (2)	>10,000 (2)
MPTH(1-14)	0.5+/-0.1 (4)[b,e] 104+/-2[d,e]	1,353+/-349 (2)*[d,e] 87+/-2[e]	21+/-3 (3)*[b] 90+/-13[e]	136+/-54* (3) 60+/-10*[e]	234+/-9 (3)* 151+/-2**
[Aib[1,3],Nle[8], Gln[10],Har[11]]PTH(1-11)NH_2	17+/-4 (4)* 101+/-1	>10,000 (2)	587+/-235* (4) 91+/-1**	>10,000 (2)	>10,000 (2)

*Statistically significant difference ($p<0.001$) in agonist potency or efficacy compared to PTH(1-34) as calculated by unpaired two-tail students t-test

** Statistically significant difference ($p<0.05$) in assay potency or efficacy compared to PTH(1-34) as calculated by a paired one-tail students t-test

Statistically different EC_{50} or efficacy ($p<0.5$) where a=cAMP accumulation assay, b=IP_1 accumulation assay, c=β-arrestin recruitment assay, d=ERK1/2 phosphorylation assay, and e=receptor internalization assay as calculated by a one-way ANOVA analysis followed by a Tukey's post-test

FIGURE 1

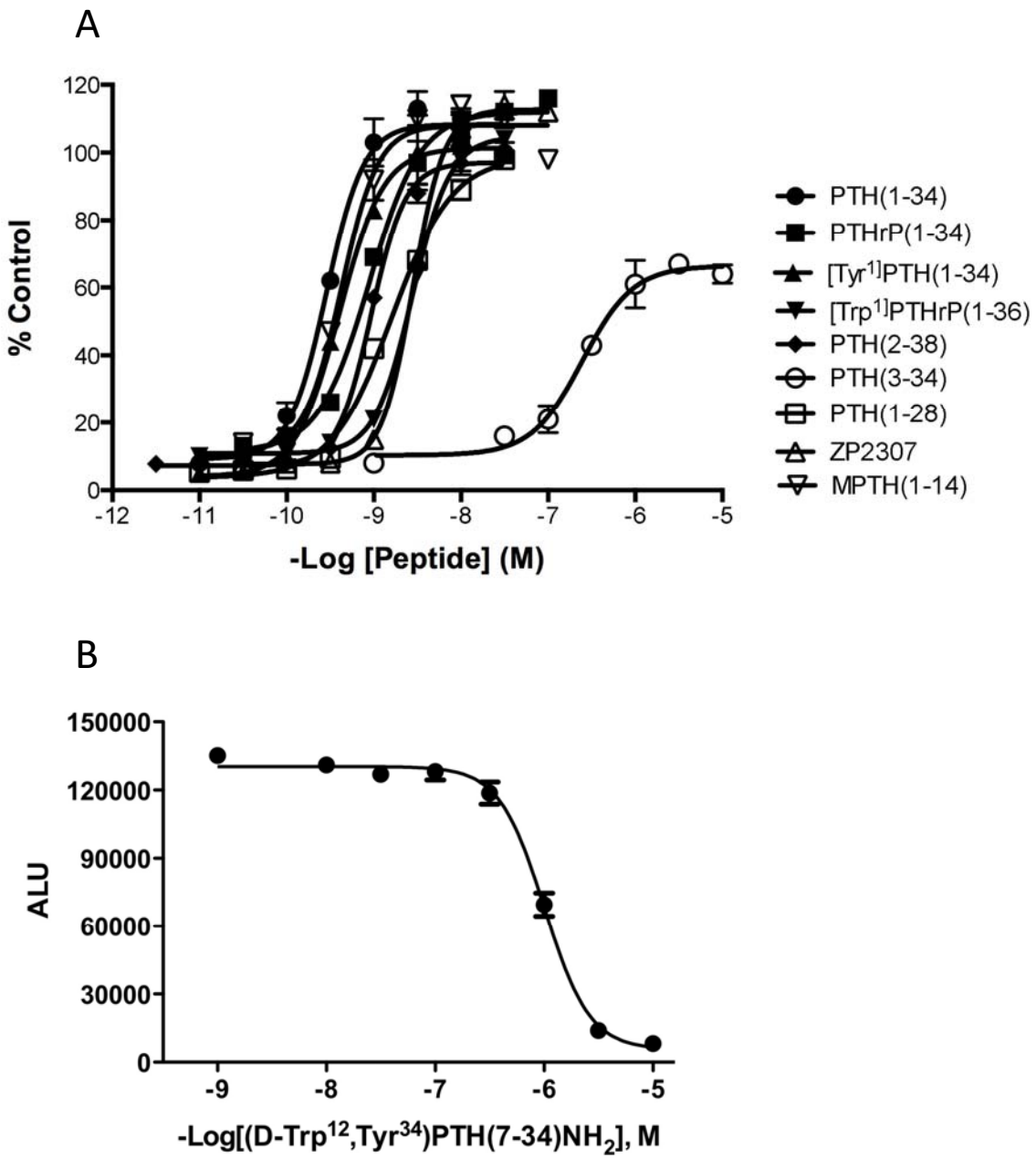


Figure 2.

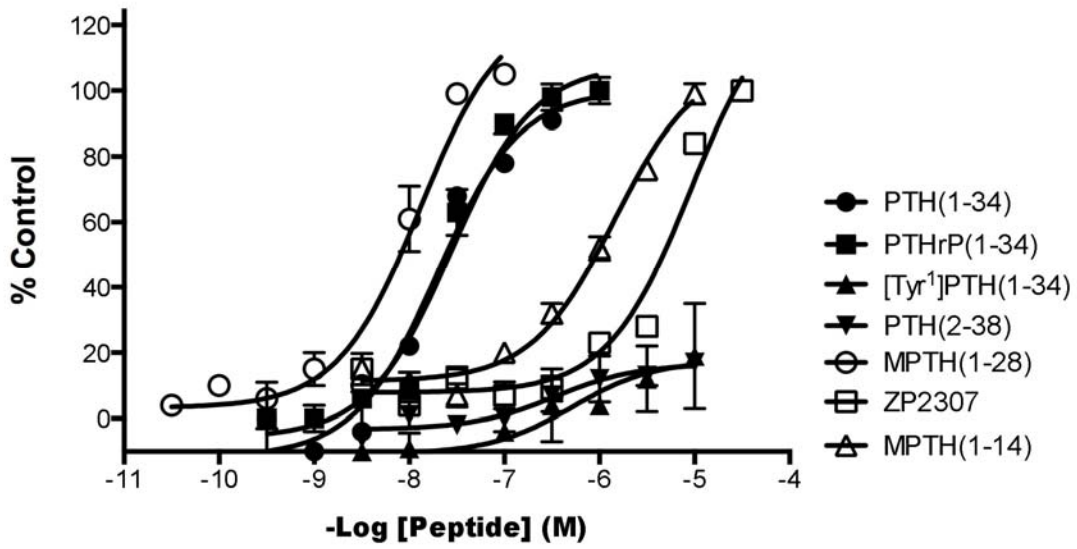
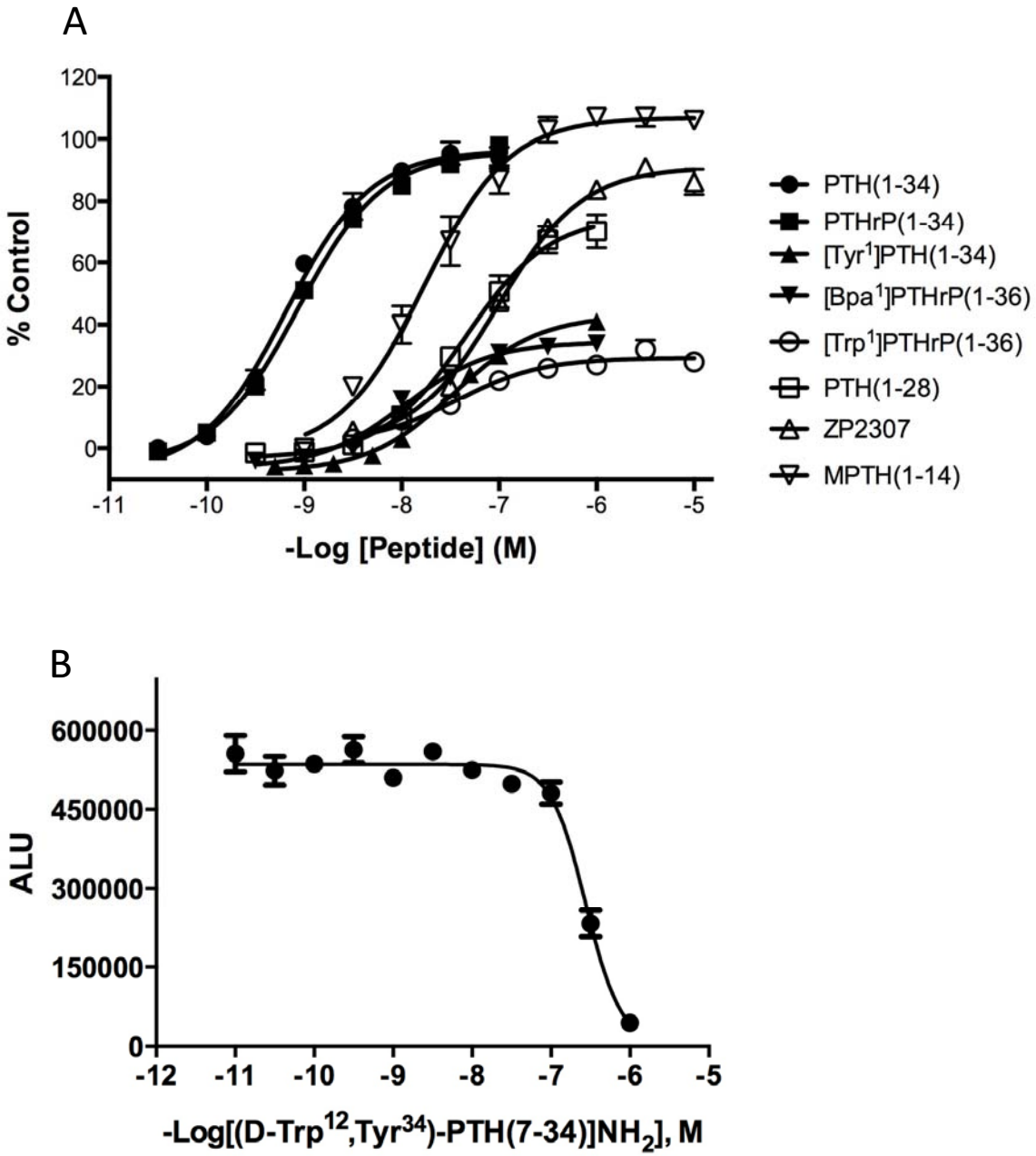


Figure 3.



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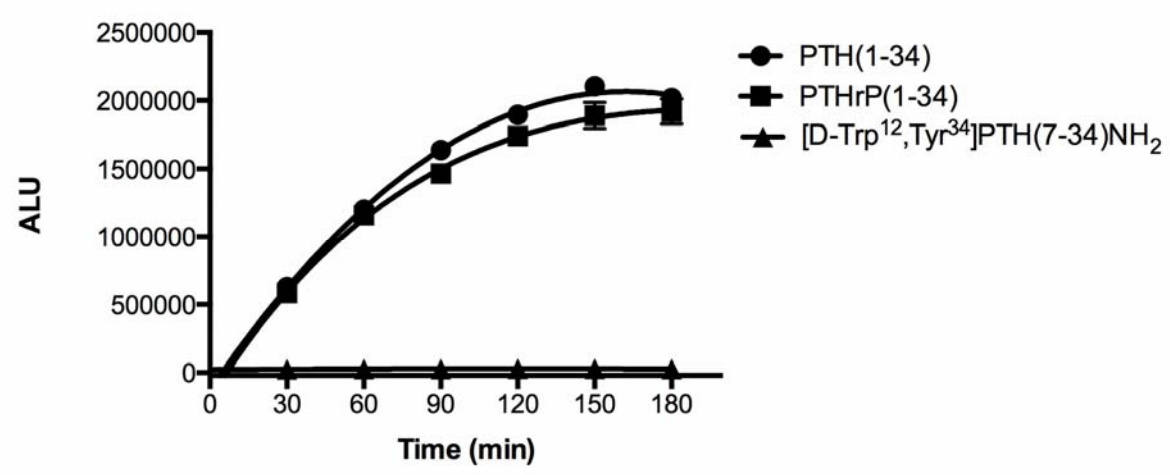

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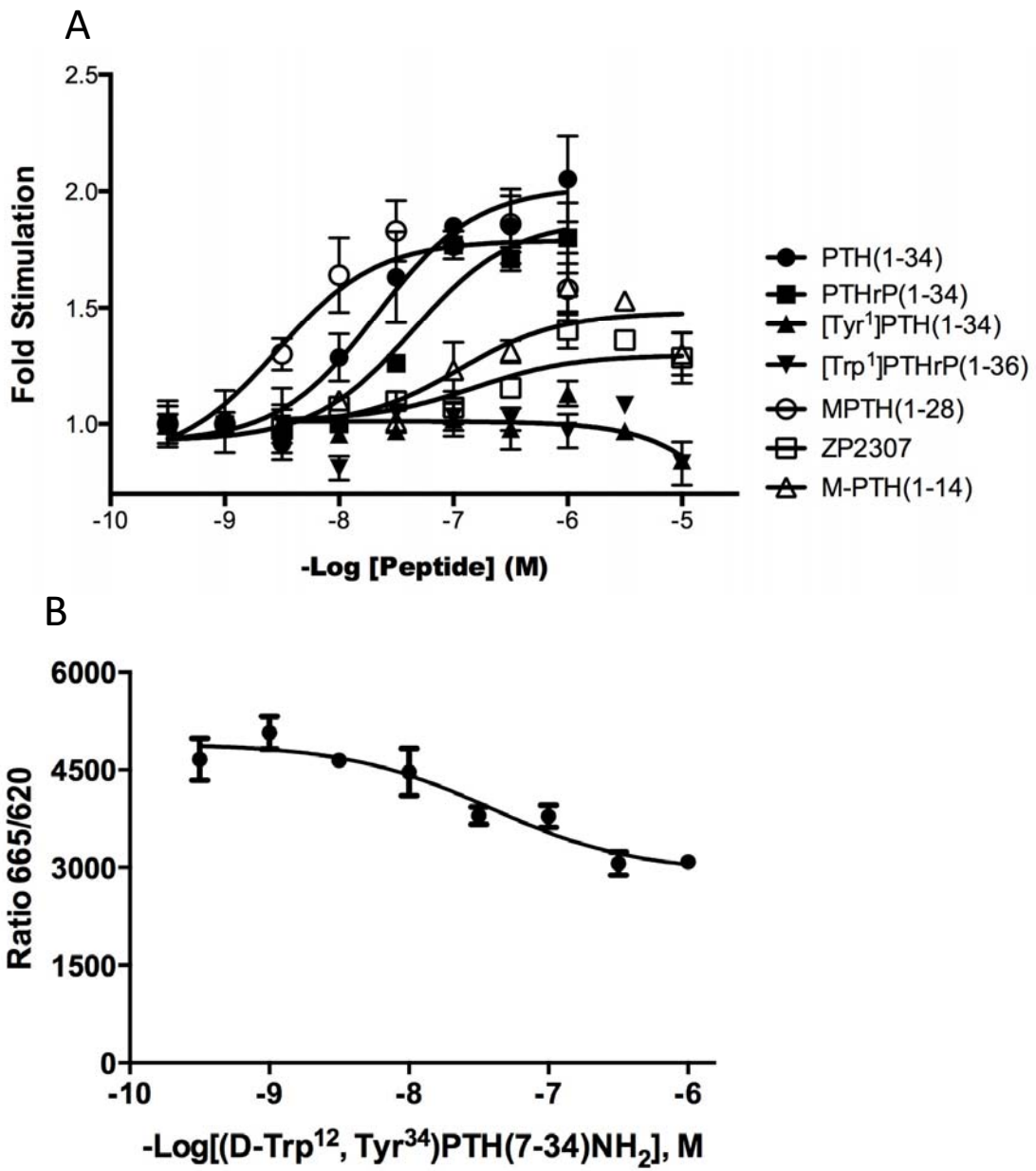

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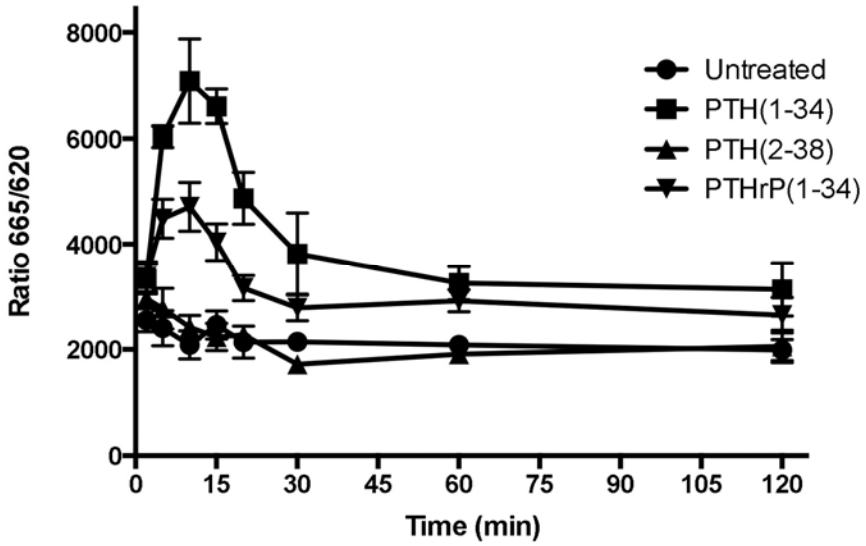

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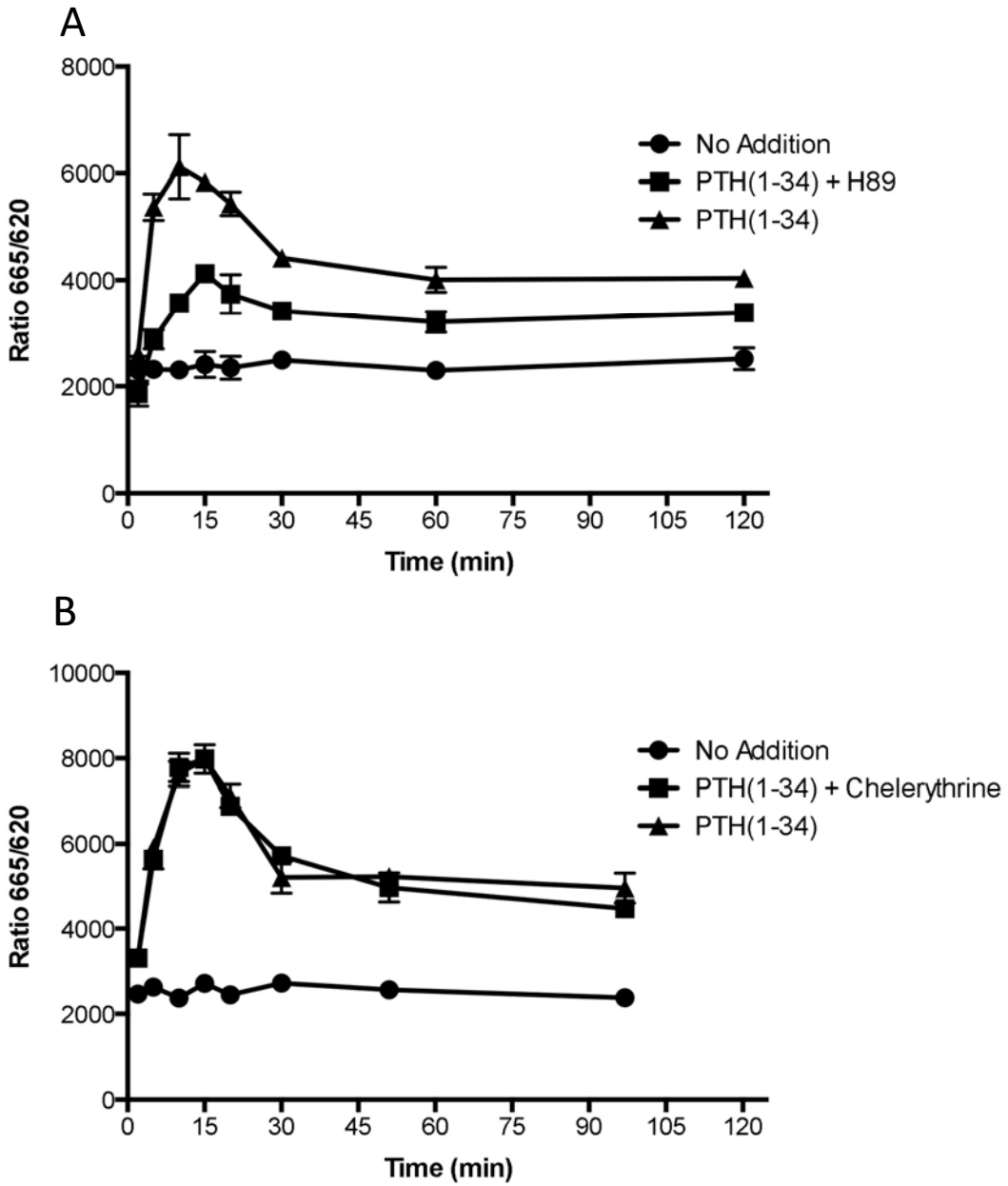

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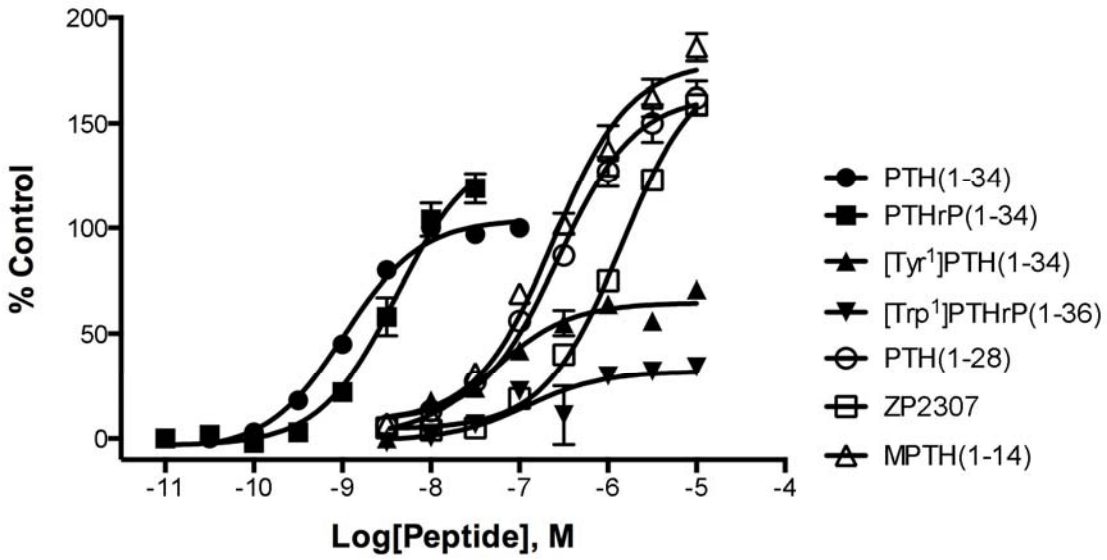

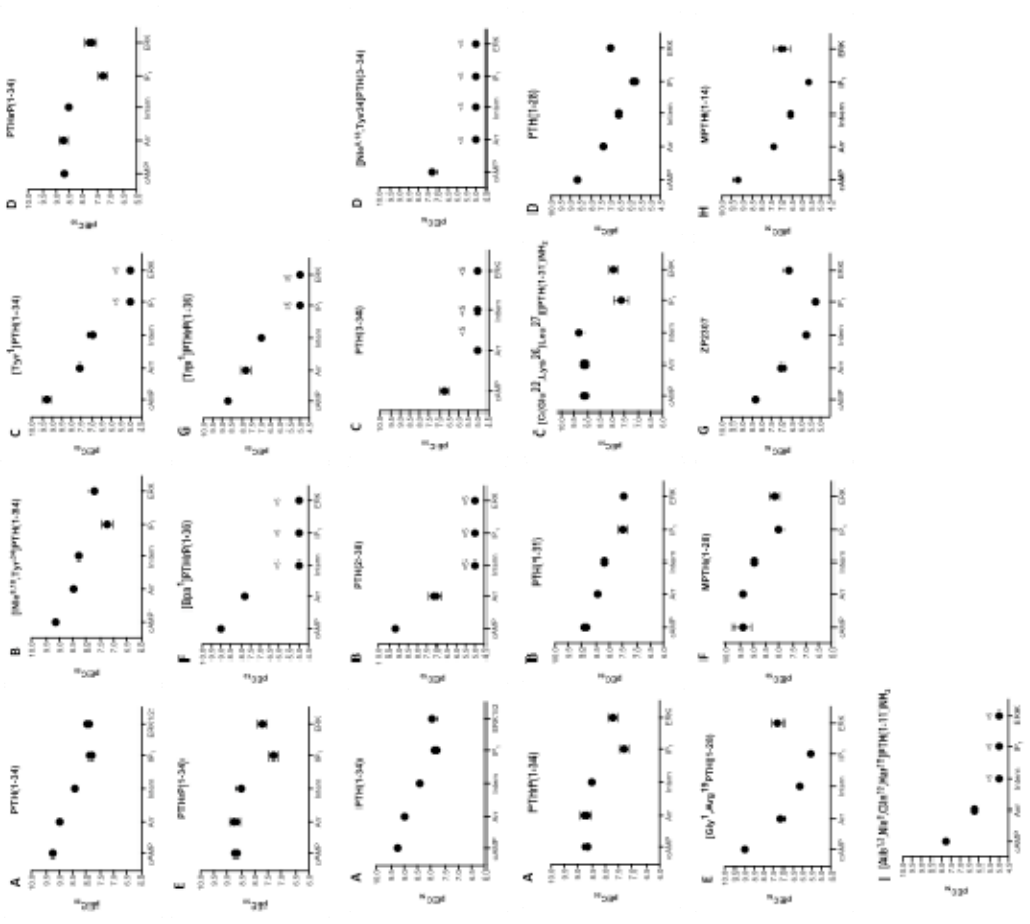

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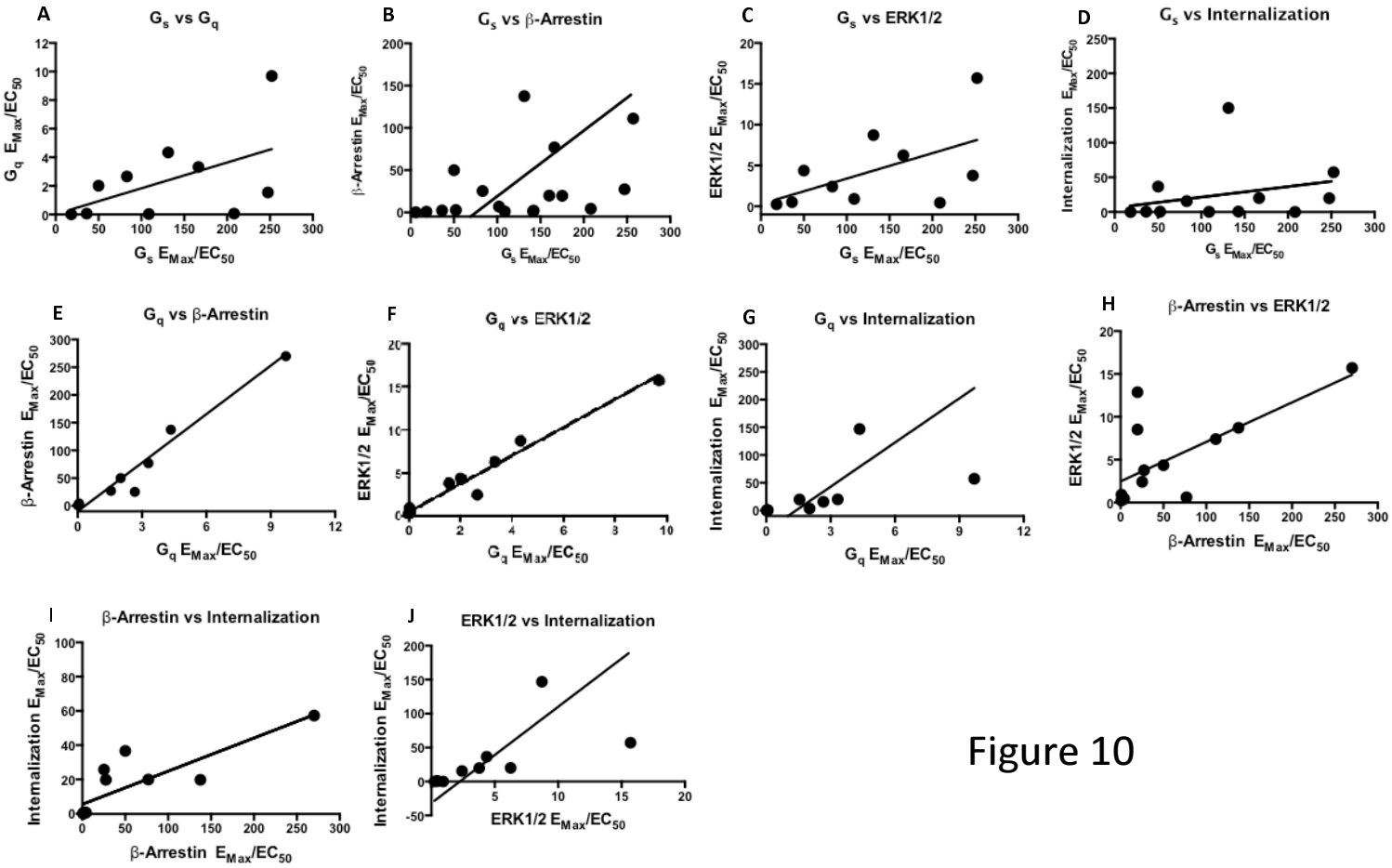

Figure 10

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Figure 11.