Improved GPCR Ligands Based on Genetically Impossible Nanobody-Peptide Fusions

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Here we describe a new method for improving signalling potency and selectivity for suboptimal GPCR ligands through conjugation with receptor-targeting nanobodies

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Improved GPCR ligands based on genetically impossible nanobody-peptide fusions.

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Abstract: Antibodies conjugated to bioactive compounds such as cytotoxic drugs allow targeted delivery of therapeutics to cell types of choice, based on that antibody's specificity. We present a new type of conjugate that consists of nanobodies and peptidic ligands for G protein-coupled receptors (GPCRs), fused via their C-termini. We address activation of parathyroid hormone receptor-1 (PTHr1), the target of teriparatide used to treat osteoporosis, which has proven refractory to potent activation by small molecules. We improve the signaling activity and specificity of otherwise poorly active N-terminal peptide fragments of PTH by conjugating them to nanobodies that recognize the extracellular portion of PTHr1. These C-to-C conjugates show biological activity vastly superior to that of the parent peptide, both *in vitro* and *in vivo*. The lead conjugate possesses selectivity for PTHr1 also superior to that of teriparatide. This platform, dubbed "conjugation of ligands and antibodies for membrane proteins (CLAMPs)", is a new approach that can generate ligands for cell surface receptors with properties superior to those provided by chemistry or nature.

Introduction: Antibodies bind tightly and specifically to their targets, even in highly complex environments. This property of antibodies has been used to deliver bioactive compounds to sites of interest, both for diagnostic and therapeutic applications¹. For example, conjugates between antibodies and cytotoxic drugs (antibody-drug conjugates or ADCs) can selectively kill cancer cells². The success of ADCs often depends on the internalization of the conjugate through endocytosis, followed by release of the cytotoxic payload. Far fewer studies have made use of antibodies to deliver bioactive compounds with sites of action at the cell surface. The conjugation of a ligand for a surface receptor to an antibody that recognizes that same receptor should increase the effective concentration of the ligand and so increase its potency and specificity, provided appropriate spatial constraints are maintained. Ideally, this method could be used with an antibody that directly targets the receptor of interest to enable application to cells and organisms without the need for their genetic modification. The G protein-coupled receptor (GPCR) family of proteins is an attractive target to pursue this approach.

Molecules that target GPCRs represent more than 25% of all approved drugs³. Antibodies and the variable fragments of camelid heavy chain-only antibodies (VHHs or nanobodies) have found increasing use for modulating GPCR signaling^{4, 5}. GPCRs and their ligands display a considerable degree of degeneracy. Several natural ligands bind to more than a single GPCR and many GPCRs can bind more than one ligand^{6, 7}. The parathyroid hormone receptors constitute one such example: a bioactive N-terminal fragment of parathyroid hormone (PTH, residues 1-34), used under the name teriparatide to treat osteoporosis, potently activates both type-1 and type-2 PTH-receptors (PTHR1/PTHR2)⁸. PTHR1/2 are part of the B-family of GPCRs which are naturally activated by large (>25 residue) peptides⁹. Despite intense pharmaceutical interest, no small molecule agonists of B-family GPCRs with potencies comparable to the natural ligands have been described. To address PTHR signaling and selectivity, we prepared conjugates of fragments of PTH and VHHs. VHHs are appealing building blocks for these

conjugates, as they are the smallest antibody fragments that retain the ability to bind antigen and can be produced in high yield using bacterial expression¹⁰. The site of antigen recognition on VHHs is near the N-terminus^{11, 12} and the interaction of PTHR1 and PTHR2 with their ligands requires a free N-terminus on the latter^{8, 13, 14}. Using a chemo-enzymatic approach we therefore made C-to-C-terminal fusions of PTH fragments and VHHs¹⁵. These adducts, dubbed “conjugates of ligands and antibodies for membrane proteins” or CLAMPs, target either wild-type or engineered receptor variants (Figure 1). These CLAMPs display biological activities *in vitro* and *in vivo* that are vastly superior to those of the PTH fragments from which they were derived. When weakly active PTH fragments are incorporated into these conjugates, they can be made exquisitely selective for activation of only those receptors engaged by the VHH. This stands in marked contrast to the lack of selectivity shown by PTH(1-34)⁸. These findings suggest that CLAMPs should be broadly applicable for the design of ligands with unique and useful properties.

Results:

Receptor constructs and conjugates used for targeting. PTH(1-34) interacts with PTHR1 via a two-site mechanism of interaction (Figure 1a-b)^{8, 16}. The association between the extracellular domain of PTHR1 and residues 12-34 of PTH provides the bulk of the binding energy and specificity for this interaction. The association between the transmembrane domain of PTHR1 and residues 1-14 of PTH induces a conformational change in the receptor, which initiates intracellular signaling cascades. This mode of interaction, supported by a large amount of structure-activity relationship data, has been confirmed recently by high resolution crystallographic and cryo-electron microscopy analysis of PTHR1-ligand interactions (Figure 1a)^{13, 14}.

To mimic receptor association exhibited by PTH(1-34), we used either wild-type PTHR1 or PTHR1 variants, modified to carry an epitope in the extracellular domain recognized by a VHH of choice. While there is no structural information for any VHH bound to PTHR1, we envisioned a mode of interaction between the receptor and VHH-PTH conjugates like that depicted in Figure 1c. The portion of PTHR1 encoded by exon 2 is not resolved in structural studies (Figure 1d)^{13, 14, 17}, is not important for ligand binding¹⁸, and in past work has been targeted as a site for receptor modification^{18, 19}. We used a receptor construct in which a green fluorescent protein variant (GFP) was inserted into the portion of the receptor encoded by exon 2 (PTHR1_{GFP} in Figure 1f)¹⁹. We also used a version of PTHR1 in which yellow fluorescent protein (YFP) replaces the entire extracellular domain (PTHR1_{YFPΔECD}, Figure 1e)²⁰. Finally, we generated a construct that encodes a PTHR1 variant in which a 14-residue fragment from exon 2 was replaced with a 14-mer epitope tag from the intracellular protein UBC6e (PTHR1_{6E}, Figure 1d)²¹.

Figure 1: Schematic of VHH-mediated delivery of PTHR ligands and the constructs used in this study. (a) Crystal structure of human PTHR1 (silver) bound to PTH(1-34) (orange) protein data bank entry (PDB): 6FJ3. (b) Structure as in panel a but with PTHR1 residues 231-296 and 349-353 shown in transparency to allow visualization of the N-terminal portion of PTH inserted into the transmembrane domain. (c) Modeled complex of PTHR1 with VHH-PTH(1-11). The VHH structure (blue) is based on VHH_{GFP} from PDB: 3K1K and PTH(1-11) bound to receptor (orange) from PDB: 6FJ3. The VHH is colored blue except for the complementarity determining loops (red), which bind the target, and the C terminus (magenta), where the PTH fragment is attached. Neither the site of binding for VHH_{PTH}, nor is its orientation relative to PTHR1 is known, as indicated by the ghost version of the VHH. Modeled structures of (d) PTHR1_{6E}, (e) PTHR1_{YFPΔECD}, and (f) PTHR1_{GFP}. (d) The predicted location of the PTHR1

segment encoded by exon 2 is highlighted in the dashed box. The orientations of the inserted tags (6E-blue, YFP-yellow, GFP-green) relative to the remainder of the receptor are not known. **(e)** Residues 31-179 from PTHR1 and residues 12-34 from PTH (PDB: 6FJ3) were removed to provide this structure. **(e-f)** PTHR1_{YFPΔECD} and PTHR1_{GFP} are depicted in complex with VHH_{GFP} (blue; PDB 3K1K). **(f)** The distance constraint imposed by introduction of the GFP/YFP-VHH pair is highlighted here.

To target these receptors, we constructed conjugates comprised of N-terminal fragments of PTH and VHHS (Figure 1). We used VHHS that recognize green or yellow fluorescent proteins (VHH_{GFP})²², a 14-mer peptide fragment from the intracellular protein UBC6e (VHH_{6E})²¹, or PTHR1 itself (VHH_{PTHR})²³. We expressed C-terminally His-tagged VHHS in bacteria in a form amenable to subsequent site-specific functionalization at the C terminus, using sortase A-mediated labeling (sortagging)^{24, 25}. To these VHHS we attached either a triglycine-modified fluorophore for cytofluorimetry or a peptide with azide and biotin moieties for biorthogonal chemistry and conjugate tracking, respectively (Figure 2).

Figure 2: Synthetic peptides and conjugation strategy. (a) Structure of synthetic peptides used in this study. Residues that differ from human PTH and are derived from the M-PTH structural series are shown in red²⁶. The residue denoted “U” corresponds to aminoisobutyric acid (Aib), depicted at right. (b) Synthetic scheme used to prepare PTH-VHH C-to-C terminal fusions. (c) Mass spectra from the preparation of VHH_{PTH}-PTH(1-11) conjugates. Complete lists of mass spectral data for peptides and conjugates are found in Supporting Figures 1 and 2.

We determined whether VHHs bound to their intended targets on live cells by flow cytometry (Figure 3a). We stained HEK293 cell lines stably transfected with the PTHR1 variants described above, rat PTHR1 (rPTH1)²⁷, or PTHR2. Each of the VHHs stained the expected cell lines, with the exception of the VHH_{GFP}-PTH1_{GFP} pair. While the VHH_{GFP} used here binds both GFP and YFP²², at 100 nM it only weakly stained HEK293 cells stably expressing PTHR1_{GFP}. This weak staining is likely related to the inability of VHH_{GFP} to tightly bind the pH-sensitive GFP variant known as pHluorin2 engrafted into the receptor¹⁹. VHH_{PTH} bound all constructs that retained the PTHR1 ECD, including rat PTHR1, but not PTHR1_{YFPΔECD}. This places the binding site primarily in the PTHR1 extracellular domain (ECD). Only VHH_{GFP} stained cells that express PTHR1_{YFPΔECD}, consistent with its ability to bind YFP²². None of the VHHs tested stained the cell line that expresses PTHR2²⁸. To estimate the affinity of the selected VHH for their targets we used flow cytometry to quantify binding. The staining of PTHR1_{YFP-del-NT} by VHH_{GFP} and PTHR1_{6E} by VHH_{6E} exhibited half-maximal staining at less than 10 nM, whereas the staining of cells expressing each of the PTHR1 receptor constructs that retained the ECD reached half-maximal staining at 100-200 nM (Supporting Figure 3).

Figure 3: Binding of VHHs to HEK293 cell lines stably transfected with PTHRs. (a) Analysis of VHH binding to PTHR1, PTHR2 and variants by flow cytometry. HEK293 cell lines in suspension were incubated on ice with 100 nM VHH sortagged with Alexafluor647, pelleted by centrifugation, washed and analyzed. (b) Analysis of VHH binding with microscopy. Adherent HEK293 cells expressing human PTHR1 were stained on ice with 50 nM VHH_{PTHR}-tetramethylrhodamine (TMR) and 30 nM PTH(1-20)-fluorescein (FAM) for 30 minutes. Following staining, cells were washed and treated with fixative in preparation for image acquisition either immediately after staining (0 m) or following a 15-minute incubation in medium at room temperature (15 m).

We used microscopy to complement these flow cytometry experiments and visualized trafficking of either PTHR1 or PTHR1_{6E} following engagement by PTH(1-20) functionalized with fluorescein and either VHH_{PTHR} or VHH_{6E} tagged with tetramethylrhodamine (Figure 3b, Supporting Figure 4). Imaging of cells fixed immediately after staining on ice shows colocalization of VHH and PTH at the cell surface. Following a 15-minute incubation at room temperature, we observed punctate and colocalized fluorescent signals, corresponding to endocytosed receptor-PTH-VHH complexes²⁹. Such colocalization indicates that the VHHs used and PTH(1-20) can simultaneously engage the receptor.

Peptide ligands of PTHR1 and conjugation to antibody fragments. To test whether delivery of PTH fragments to their site of action by conjugation to VHHs affects their signaling activity we synthesized N-terminal fragments of PTH (Figure 2, Table 1, Supporting Figure 5). These fragments were prepared as C-terminal amides by conventional solid-phase peptide synthesis, purified, and their identities were confirmed by mass spectrometry (Figure 2, Supporting Figure 1). Most of these peptides contained several of the modifications found in the M-PTH series of peptides, including the non-standard residue aminoisobutyric acid (Aib) at position 3, which enhances the biological activity of these short PTH fragments (Figure 2)²⁶. Each of these peptides contained a C-terminal cysteine (Cys). Using Cys-maleimide chemistry we appended a dibenzylcyclooctyne (DBCO) handle (Supporting Figure 1) to enable an azide-alkyne conjugation between the C-termini of azide-functionalized VHHs and DBCO-modified synthetic peptides. The composition of the conjugates was confirmed by mass spectrometry (Figure 2,

Supporting Figure 2). We also prepared conjugates in which a PTH(1-14) analogue with an N-terminal triglycine extension (G₃-PTH(1-14)) was conjugated to VHHs using sortagging (Supporting Figure 6).

We then assessed the capacity of these peptides and conjugates to stimulate the production of cyclic adenosine monophosphate (cAMP), a second messenger molecule produced upon PTHR1 activation, using HEK293 cells expressing a targeted PTHR variant and a luciferase-based cAMP-responsive reporter³⁰. Progressive truncation of C-terminal residues from PTH(1-34) caused a marked loss in the potency on wild-type PTHR1 and other PTHR1 variants with intact ECDs (Table 1, Supporting Figure 5). Addition of a triglycine appendage at the N-terminus of PTH(1-14) caused a reduction in potency, relative to PTH(1-14) with a free N-terminal amine (Supporting Figure 6), in line with precedent³¹. Conjugates in which G₃-PTH(1-14) was ligated to the VHH C-terminus using sortase were completely inactive, emphasizing the importance of a free N-terminus for PTH (Supporting Figure 6). In contrast, conjugates formed by C-to-C-terminal fusion were active (Table 1, Supporting Figure 5).

Peptide or conjugate	hPTHR1 (nM ± SD)	PTHR1 _{GFP}	PTHR1 _{6E}	hPTHR1 _{YFPΔECD}
PTH(1-34)	0.51 ± 0.28	0.4 ± 0.8	1.3 ± 1.0	689 ± 301
PTH(1-14)	4.3 ± 2.0	2.3 ± 1.2	3.4 ± 1.6	1.1 ± 0.9
PTH(1-11)	516 ± 238	79 ± 45	94 ± 74	246 ± 133
PTH(1-10)	3121 ± 1671	2552 ± 653	5079 ± 407	3841 ± 1604
PTH(1-9)	Inactive at 10,000 nM	Inactive at 10,000 nM	Inactive at 10,000 nM	Inactive at 10,000 nM
VHH _{PTHR} -PTH(1-14)	0.075 ± 0.041	0.5 ± 0.2	0.2 ± 0.1	0.9 ± 0.5
VHH _{PTHR} -PTH(1-11)	5.0 ± 1.6	0.5 ± 0.1	4.0 ± 3.2	Inactive at 100 nM
VHH _{PTHR} -PTH(1-10)	Inactive at 100 nM	ND	ND	ND
VHH _{6E} -PTH(1-14)	Inactive at 330 nM	ND	0.4 ± 0.2	ND
VHH _{6E} -PTH(1-11)	Inactive at 100 nM	Inactive at 100 nM	6.9 ± 2.6	Inactive at 100 nM
VHH _{6E} -PTH(1-10)	ND	Inactive at 100 nM	2.8 ± 1.4	ND
VHH _{6E} -PTH(1-9)	ND	ND	Inactive at 100 nM	ND
VHH _{GFP} -PTH(1-14)	Inactive at 100 nM	1.8 ± 0.6	Inactive at 100 nM	0.58 ± 0.29
VHH _{GFP} -PTH(1-11)	Inactive at 100 nM	8.0 ± 3.4	Inactive at 100 nM	0.14 ± 0.06
VHH _{GFP} -PTH(1-10)	ND	Inactive at 100 nM	ND	0.46 ± 0.22
VHH _{GFP} -PTH(1-10)	ND	Inactive at 100 nM	ND	~40% activation at 100 nM ⁹

Table 1: Stimulation of PTHR1 and variants by VHH-PTH conjugates. HEK293 cell lines were treated with varied doses of the indicated peptides or conjugates and activation was assessed by measuring luminescence from a cAMP-activated luciferase variant. Values listed represent EC₅₀ values (mean ± SD). Each value comes from ≥ 3 independent experiments. Further details, including the number of replicates for each measurement and the normalized maximal responses induced, are reported in Supporting Table 1. “ND” indicates that the

measurement was not made. “Inactive” indicates that the luminescence response measured at that concentration was less than 5% of the maximal response induced for that cell line. ^aThe precise EC₅₀ value could not be obtained but at the highest concentration tested (100 nM) this compound induced a response approximately 40% that of the maximal response observed.

The conjugation of PTH fragments lacking residues 15-34, known to be important for ECD binding, to VHHs that bound to the targeted receptor enhanced potency (Table 1). VHH_{6E}-PTH(1-10) is 1,800-fold more potent than PTH(1-10) on PTHR1_{6E}, and VHH_{GFP}-PTH(1-10) is 7,800-fold more potent than PTH(1-10) on PTHR1_{YFPΔECD}. The potency of VHH_{GFP}-PTH(1-10) at PTHR1_{YFPΔECD} (EC₅₀ ~ 0.5 nM) is especially notable given that PTH(1-34), an analogue with properties similar to naturally occurring PTH, is relatively weakly active (EC₅₀ >500 nM) on this receptor. VHH_{GFP}-PTH(1-9) is active at 100 nM on PTHR1_{YFPΔECD}, whereas PTH(1-9) itself was inactive at 10,000 nM on all constructs tested. Similar trends are seen with larger PTH fragments. VHH_{6E}-PTH(1-11) is 23-fold more potent than PTH(1-11) at PTHR1_{6E} and VHH_{GFP}-PTH(1-11) is 1,800-fold more potent than PTH(1-11) at PTHR1_{YFPΔECD}. VHH_{PTHR} conjugation also increases the potency of PTH(1-11) and PTH(1-14) at PTHR1, PTHR1_{GFP}, and PTHR1_{6E}, in line with results from VHH binding experiments. Even VHHs that showed weak staining of the relevant cell lines, like that of VHH_{GFP} on PTHR_{GFP} expressing cells, still enhanced the signaling activity of N-terminal fragments like PTH(1-11) upon conjugation. VHH_{GFP}-PTH(1-11) is approximately 8-fold more potent than PTH(1-11) in activating PTHR_{GFP} (Table 1). This contrasts with the lack of signaling activity of VHH_{GFP}-PTH(1-10) on PTHR1_{GFP} and VHH_{PTHR}-PTH(1-10) on PTHR1, which likely relates to combining weakly active PTH fragments with VHHs that bind to receptors with lower affinity. Signaling duration, as assessed using a previously validated method^{26, 28, 32}, is also prolonged for the shorter PTH fragments upon their conjugation to the appropriately specific VHH (Supporting Figure 7). The kinetics of the cAMP signaling induced by some VHH-PTH fragments resemble that seen with PTH(1-34) (Supporting Figure 7).

In contrast to the increase in signaling activity provided by the conjugation of PTH fragments with receptor binding VHHs, conjugation of active PTH fragments with irrelevant VHHs is detrimental. For example, conjugates of VHH_{6E} and VHH_{GFP} with PTH(1-11) and PTH(1-14) are inactive on wild-type human PTHR1 at the highest concentrations tested, even though the peptides themselves are quite active (Table 1, Supporting Figure 5). This loss of activity is caused at least in part by a loss in receptor binding for PTH fragments conjugated to irrelevant VHHs (Supporting Figure 8). VHH_{6E}-PTH(1-14) fails to bind hPTH1R expressing cells whereas VHH_{PTH1R}-PTH(1-14) binds more tightly than VHH_{PTH1R} alone. The impact of irrelevant VHH conjugation is not explained by variation in signaling activity caused by the chemical handles installed for azide-alkyne conjugation chemistry (Supporting Figure 9). Furthermore, the length of the VHH-PTH linker is not a strong determinant of conjugate signaling activity or specificity: incorporation of a PEG₃ linker has minimal impact (Supporting Figure 9). The enhancements in signaling activity provided by VHH conjugation is not seen with PTHR1 ligands that retain their ECD binding element: conjugates of PTH(1-34) and VHHs retain potent biological activity regardless of whether the target of the VHH is present on the cell line tested, at least at receptors with the ECD present (Supporting Figure 10). VHH_{PTH1R}-PTH(1-14) showed similar behavior at PTHR1_{YFPΔECD} (Table 1).

The increase in biological activity and selectivity of PTH fragments seen through conjugation to VHHs led us to test whether the same enhancements in activity applied to two naturally occurring subtypes of PTHR. PTH(1-34) tightly binds and activates both PTHR1 and PTHR2⁸. VHH_{PTH1R} binds to PTHR1 but not PTHR2 (Figure 3). VHH_{PTH1R}-PTH conjugates should therefore activate PTHR1 but not PTHR2. We focused on conjugates of PTH(1-14) as this fragment also activated PTHR1 and PTHR2 (Figure 4). The VHH_{PTH1R}-PTH(1-14) conjugate activated PTHR1 more potently than any other compound tested in this study ($EC_{50} \sim 0.07$ nM), whereas it was completely inactive at PTHR2 at 330 nM (>4,500-fold selectivity for PTHR1, Figure 4). This

contrasts with the lack of selectivity of PTH(1-34) in this study (5-fold selectivity for PTHR1) and in past work⁸.

Figure 4: Selective and potent activation of PTHR1 via VHH_{PTH1} conjugation. HEK293 cell lines stably expressing either human PTHR1 (hPTH1) or hPTH2 were treated with varied doses of the indicated peptides or conjugates and activation was assessed by cAMP production. **(a-b)** Representative dose-response curves for **(a)** hPTH1 or **(b)** hPTH2 activation. Data points indicate mean ± SD. Curves result from fitting of a sigmoidal dose-response model to data. **(c)** Tabulation of cAMP induction potencies. Data for hPTH1 are identical to those in Table 1 and are included here for comparison. Values listed represent EC₅₀ values (mean ± SD). Each value comes from ≥ 3 independent experiments. Further details, including the number of replicates for each measurement (n), are reported in Supporting Table 1.

In vivo activity. We tested whether the potent biological activity observed for VHH-PTH conjugates in cell-based assays would extend to an *in vivo* setting. We used the VHH_{PTH1}-PTH(1-14) conjugate in these experiments because, unlike VHH_{PTH1}-PTH(1-11), it stimulated signaling through the Gq/PLC signaling pathway in cells expressing human PTHR1 (Supporting Figure 11). Indeed, VHH_{PTH1} also potentiated PTH(1-11) signaling activity for the rat PTHR1 (Supporting Figure 12). Since VHH_{PTH1} bound rat PTHR1 (Figure 3) we were confident that it would also bind mouse PTHR1, as these receptors are quite similar (99% identical in their extracellular domain). To measure *in vivo* activity, we injected mice subcutaneously with equimolar amounts of either PTH(1-34), M-PTH(1-14), VHH_{PTH1}-PTH(1-14), or saline. PTH(1-

34) induces a strong increase in blood ionized calcium levels, which peaks 1-2 h after injection and returns to baseline thereafter, whereas free M-PTH(1-14) exhibits little if any activity in this assay, in line with past findings²⁶. Our experiment showed that VHH_{PTH}-PTH(1-14) stimulated a spike in blood calcium that peaked two hours after injection (Figure 5). The conjugation of PTH(1-14) with VHH_{PTH} therefore potentiates biological activity both in cell-based assays and *in vivo*.

Figure 5. VHH conjugation potentiates an *in vivo* response. (a) Schematic of the experiment performed in mice. (b) Measurement of blood ionized calcium levels in mice injected with PTH and conjugates. Mice (CD1 females, 11 weeks) were injected subcutaneously with the indicated ligand (Dose = 35 nmol/kg). Blood was drawn at the indicated time points and analyzed for ionized calcium levels. Data points indicate mean $\pm$ standard error of the mean (SEM), $n=4$, * $p<0.05$ vs. vehicle.

Discussion: Antibodies as part of conventional antibody-drug conjugates deliver cytotoxic compounds that typically target intracellular proteins^{1, 2}. Less explored is the use of antibodies to deliver ligands for surface receptors, such as GPCRs. This is likely due in part to complications in preparing homogenous and bioactive conjugates of antibodies and ligands that rely on antibody binding to potentiate engagement of receptor by the ligand. The immunocytokines (conjugates of cytokines and antibodies) are an exception³³. Immunocytokines have been characterized in cell- and animal-based models and have progressed to the clinic, but not without toxicity, suggesting an insufficiency in targeting³³. In one case, even the identity of the targeting antibody of the immunocytokine was irrelevant for in vivo efficacy³⁴. The introduction of mutations in cytokines to improve the selectivity of immunocytokines can dampen the affinity of cytokines for their receptors^{35, 36}. Immunocytokine-based approaches differ from the CLAMP platform in that they rely on genetic fusions (and genetically encoded residues) and the use of full-size cytokine domains, as opposed to the small peptide fragments with non-natural residues used here.

In one precedent for targeting GPCRs with antibody-ligand fusions, VHH_{GFP} equipped with a SNAP-tag was linked to a photoactivatable ligand for the GPCR mGluR2³⁷. This fusion was then used to activate a GFP-tagged receptor upon photoactivation of the ligand. The response induced by a saturating solution of the photoactivatable VHH-ligand conjugate was ~40% as strong as that induced by a saturating solution of natural ligand and moreover required the use of a receptor-GFP fusion³⁷. This precluded the use of genetically unmodified cells or animals. In another example, full-length anti-PKSK9 antibodies fused at the N-terminus of the heavy or light chain with analogues of the glucagon-like peptide 1 (GLP-1) were produced³⁸. However, most of the fusions tested were expressed in low yield, isolated with inactivating truncations in the GLP-1 fragment, unstable in solution, or were degraded rapidly *in vivo*, demonstrating the difficulties

encountered when expressing fusion proteins comprised of full-length antibodies and ligands of interest³⁸.

Despite several screening campaigns, no VHHs that directly activate GPCRs have been identified⁵. We prepared a library of C-to-C terminal fusions of VHHs and synthetic PTH peptides. The necessity for the generation of C-to-C fusions is supported by the lack of activity of the corresponding N-to-C fusions (Supporting Figure 6). A further benefit of our synthetic strategy is the ease of incorporation of non-natural residues such as Aib into the synthetic peptide portion of the conjugate to improve proteolytic stability³⁸. Several conjugates stimulated cAMP responses with potencies similar to that of PTH(1-34) (Table 1). Even PTH(1-9), which fails to activate PTHR1 unless tethered directly to the receptor's N-terminus via genetic fusion³⁹, showed activity when conjugated to a VHH (Table 1). VHH-mediated delivery of ligands should enable identification of weak ligands that might otherwise be dismissed as completely inactive. Similar observations were made in evaluating conjugates consisting of peptide fragments derived from the N- and C-termini of corticotrophin releasing factor-1, which were weakly active or inactive alone, but once assembled via click chemistry, several conjugates were potent agonists⁴⁰.

Agonist activity for VHH-PTH conjugates was completely dependent on binding of the VHH to the receptor being targeted: a mismatch between specificity of the VHH and the receptor construct led to a loss in conjugate activity. We identified a conjugate, VHH_{PTHR}-PTH(1-14), with very potent signaling activity in cell-based assays (Table 1), with biological activity in mice (Figure 5), and with selectivity for PTHR1 over PTHR2 that far surpasses the selectivity of PTH(1-34), the prototypical PTHR1 agonist (Figure 4) used clinically. Success in targeting PTHR1 over PTHR2 sets the stage for designing ligands that specifically activate other receptors with overlapping specificities^{6, 7}.

The ability to deliver ligands to specific subtypes of receptors, or to receptors engineered to contain an antibody-recognized tag, should allow the creation of (modular) versions of designed receptors exclusively activated by designer drugs (DREADDs)⁴¹. Previously described DREADDs for GPCRs were identified through modification of the ligand binding site of naturally occurring GPCRs, so that the modified receptors respond to a “designer” small molecule but not the ligand of the prototype receptor. These designer molecules selectively activate the designer receptor but not any endogenously expressed alternative^{42, 43}. A similar approach has been deployed to produce an orthogonal receptor-ligand pair for interleukin-2⁴⁴. Our finding that VHH_{GFP}-PTH(1-11) potently activates PTHR1_{YFPΔECD} (EC₅₀ ~ 0.15 nM) but is inactive at wild-type PTHR1, suggests a path towards using VHH-tag recognition as a way to convert a GPCR of choice into a DREADD. One aspect of GPCR pharmacology that has not been faithfully reproduced in some DREADD constructs is that of ligand binding kinetics⁴⁵. For some receptors, such as PTHR1, the duration of ligand binding and the signaling induced as a consequence can dictate the type of physiological response evoked. The duration of the cAMP response elicited by PTHR1 activation is correlated with the strength of the calcemic response *in vivo*^{26, 46}. Several of the VHH-PTH conjugates tested here induce cAMP signaling that is prolonged relative to the free peptide and similar to that of PTH(1-34) (Supporting Figure 7), suggesting that the affinity provided by VHH binding can be used as an independent means to adjust ligand binding and signaling kinetics.

In conclusion, we show that the conjugation of suboptimal PTHR1 agonist peptides to VHHS that target the intended receptor provides a substantial increase in agonist potency and receptor selectivity. The ability to independently modulate receptor affinity. The structure of the agonist used to activate signaling should enable a further dissection of connections between ligand affinity, receptor signaling kinetics, and ligand bias⁴⁷. The CLAMP platform is amenable to

targeting other GPCRs, especially those with large peptide ligands that bind to their receptors via a two-site mechanism, such as family B GPCRs and chemokine receptors.

ONLINE METHODS.

General. HEK293 cell lines were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin. Cell lines were routinely tested mycoplasma infection. LC/MS was performed on a Waters Xevo Q-ToF system equipped with HPLC-C8 columns. Mass spectra were obtained using Q-ToF mass spectrometry with a positive ionization mode. Masses for VHHs and conjugates were calculated via analysis of multiply charged ions using the MaxEnt feature on MassLynx software. Protein and peptide concentrations were calculated using absorption at 280 nm for VHHs and peptides with tryptophan (Trp) residues. For peptides without Trp, the amount of peptide was quantified gravimetrically assuming that the weighed mass consisted of 50% peptide (w/w).

Plasmids and DNA. Cell lines stably expressing human PTHR1, rat PTHR1, PTHR1_{GFP}, and PTHR1_{YFPΔECD} along with a cAMP-responsive luciferase variant have been previously reported^{19, 20, 27}. PTHR1_{6E} was produced using the Q5 Site-directed mutagenesis kit (NEB). Annotated sequence data for PTHR1_{6E} and alignment with hPTH1R is found in Supporting Figure 13. The sequence of PTHR1_{YFPΔECD} is shown in Supporting Figure 14. Materials are available upon request.

Peptide synthesis. Peptides were prepared using conventional solid-phase synthesis methods with Fmoc-protection of backbone amines. Synthesis was performed on Rink-amide linker resin to yield C-terminal amides. Backbone deprotection was performed via treatment with piperidine in dimethylformamide (DMF, 20% vol/vol) for 15 minutes at room temperature. Coupling was performed using Fmoc-protected amino acids (4 equivalents), N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 4 equivalents), and

diisopropylethylamine (DIPEA, 8 equivalents) in DMF for 45 minutes at room temperature. Fmoc-Lys(biotin)-OH and Fmoc-Lys(azide)-OH were from used from commercial sources without alteration the synthetic methods described above. Following completion of synthesis, the resin was dried and deprotection was carried out using a solution of 92.5% trifluoroacetic acid, 5% H₂O, and 2.5% TIPS. Peptides were precipitated into diethyl ether, pelleted by centrifugation, dried under a stream of air, purified using reversed-phase C18 HPLC using a water-acetonitrile gradient, and lyophilized. The identity and approximate purity of peptides was confirmed by LC/MS (Supporting Figure 1). Purified products were dissolved in water (10 mM stock concentration) and stored at -20 °C.

Purified peptides with C-terminal cysteines were subjected to a reaction with a 2-fold molar excess of either DBCO-maleimide (Click Chemistry Tools) or DBCO-PEG₃-maleimide (ConjuProbe) (Supporting Figure 9) in solvent with 50% (v/v) dimethylsulfoxide (DMSO) and 50 mM pH 7.4 phosphate buffer and purified by reversed-phase C18 HPLC. The identity of peptides was confirmed by LC/MS (Supporting Figure 1). Purified products were dissolved in DMSO (1 mM stock concentration) and stored at -20 °C.

Protein expression and purification. The production and purification of VHH_{GFP} (named VHH-enhancer) and VHH_{6E} (named VHH05) has been described previously^{21, 22}. The sequence for VHH_{PTHR} was acquired from the literature (named 22A3)²³. Briefly, VHs were expressed using the pHEN6 vector. Plasmids coding for PelB-VHH-LPETGG-His₆ were transformed into WK6 *E. coli* using heat shock. Transfected WK6 *E. coli* were grown in Luria Bertani broth under ampicillin selection at 37 °C until an optical density at 600 nm between 0.6 and 0.8 was reached. Protein expression was induced by the addition of 1mM IPTG and cells were grown at 30 °C overnight. The bacteria were pelleted by centrifugation and resuspended in TES buffer (50 mM Tris, 650 μM EDTA, 2 M sucrose, 15 mL buffer per liter of culture) to prepare for osmotic shock. After incubating for 2 hours at 4 °C, 75 ml distilled H₂O was added, and the

bacterial suspension was incubated overnight at 4 °C. The bacteria were again pelleted and VHHs were purified from the supernatant by Ni-NTA bead batch purification, followed by buffer exchange. Sortase-A pentamutant was expressed and purified as previously described³.

Flow cytometry. Suspensions of cells in PBS were stained for 1 hour on ice in the presence of indicated concentrations of VHH probes functionalized with Alexafluor647. Cells were pelleted by centrifugation and washed with PBS prior to analysis by flow cytometry (BD Accuri C6). Gating was performed on forward scatter/side scatter profiles to analyze intact cells. Data was analyzed using FlowJo version 7.6. The median fluorescent intensity (MFI) of stained cells was used to generate VHH binding dose response curves (Supporting Figure 3).

Sortase-mediated labeling (sortagging). VHHs were labeled using sortase A pentamutant as described²⁵. Briefly, VHH (20-100 µM) with a C-terminal sortase-recognition motif and His-tag were incubated with GGG-peptide (500 µM) and sortase A pentamutant (10 µM) in Tris-buffered saline (TBS) containing 10 mM CaCl₂ overnight at 14° C. Functionalized VHHs were purified from unreacted VHH and sortase by exposure to nickel-NTA sepharose beads and removal of GGG-peptide by buffer exchange using a 10 kDa molecular weight cutoff spin filter or a PD10 disposable size exclusion column. Purified VHH conjugates were concentrated using 10 kDa spin filter. VHH-PTH(1-14) and VHH-G₃-PTH(1-14) conjugates were prone to precipitation following concentration.

VHH-peptide conjugation reactions. VHH-biotin-azide conjugates (Figure 2) were mixed with PTH-DBCO (3-fold molar excess) in TBS with 10% (v/v) glycerol. The reaction was shaken at 22° C until unreacted VHH-biotin-azide had been completely consumed. The product conjugate was purified from free PTH-DBCO using a PD10 size exclusion column. Product identity was confirmed by LC/MS (Supporting Figure 2).

Microscopy. Monolayers of HEK293 cells grown on glass cover slips at approximately 80% confluency expressing either hPTHr1 or PTHR_{6E} were washed with Hanks balanced salt solution supplemented with 10 mM HEPES pH 7.4 and 0.1% (w/v) bovine serum albumin (HB). The cells were then stained with M-PTH(1-20)-fluorescein and VHH-tetramethylrhodamine in HB at 4° C for 30 m. After staining, cells were washed with HB three times, and fixed with 4% formalin either immediately after rinsing or following a 15 m incubation at room temperature in DMEM + 10% FBS. Cells were then rinsed and mounted with Vector-shield containing DAPI (to visualize nuclei) on glass slides for imaging. Images were acquired using a Nikon Eclipse Ni system with a 40x PLAN FLUOR 0.75NA DIC M/N2 objective.

Measurement of cAMP response. These assays were performed as previously described²⁷. Briefly, HEK-293-derived cell lines that stably express the Glosensor cAMP reporter (Promega Corp.)³⁰ and PTHR1, a PTHR1 variant, or PTHR2 were seeded into white sided 96 well plates (50,000 cells/well) and grown to confluency. Confluent monolayers of cells were pre-incubated with CO₂ independent medium containing D-luciferin (0.5 mM) at 37° C until a stable baseline level of luminescence was established (20 min). Varying concentrations of ligands were then added, and the time course of luminescence response was recorded using BioTek plate reader. The maximal luminescence response (observed 12–16 min after ligand addition) was used to construct dose-response data sets (Table 1, Supporting Figure 5).

For the measurement of cAMP signaling duration experiments (Supporting Figure 7) were performed as previously described²⁸. Cells were treated with ligands at the indicated concentrations for 12 minutes (ligand-on phase). After this period, the medium in each well was removed and the cells were rinsed twice with CO₂-independent medium to remove unbound

ligand. After the addition of D-luciferin-containing fresh medium to each well, the luminescence was recorded for an additional 30-40 minutes (ligand-off phase).

Measurement of cytoplasmic calcium mobilization. The mobilization of Ca^{2+} levels was assessed in the HEK293 cell line stably transfected with human PTHR1. Intracellular Ca^{2+} levels were assessed using a cell-permeant Ca^{2+} sensor, Fura2-AM (Invitrogen). Cells in a black 96-well plate were loaded with Fura2-AM in the presence of Pluronic F-127 for 45 min and then rinsed with Hanks buffered saline solution (HBSS). Following an additional 30-minute incubation in HBSS, the plate was analyzed using a PerkinElmer Life Sciences Envision plate reader to monitor fluorescence emission at a wavelength of 515 nm, upon excitation at wavelengths of 340 and 380 nm. The data were recorded at 2-second intervals prior to and after ligand addition. The data were calculated as the ratio of the fluorescence signal obtained with excitation at 340 nm to that obtained with excitation at 380 nm.

Animal experiments and measurement of in vivo response. Mice (CD1 female, age 11 weeks) were treated in accordance with the ethical guidelines adopted by Massachusetts General Hospital. Calcemic response assays were conducted using cohort sizes comparable to past work²⁷, which provided data adequate for identifying differences in the time course and magnitude of PTH-induced calcemic responses. Statistical analyses were performed assuming Gaussian distribution of data. Mice ($n = 4$ per compound) were injected subcutaneously with vehicle (10 mM citric acid/150 mM NaCl/0.05% Tween-80, pH 5.0) or vehicle containing PTH or conjugate at a dose of 35 nmol/kg body weight. Prior to injection, mice were grouped according to basal blood calcium concentrations to ensure each group possessed similar average (mean) blood ionized calcium levels at $t = 0$. Blood was withdrawn just before injection ($t = 0$) or at times thereafter. Tail vein blood was collected and immediately analyzed. Blood Ca^{2+} concentration was measured with a Siemens RapidLab 348 Ca^{2+} /pH analyzer.

Data calculations. Data were processed using Microsoft Excel and GraphPad Prism 6. Data from cAMP dose–response assays were analyzed using a sigmoidal dose–response model with variable slope. Data sets were statistically compared by using Student’s t test (two-tailed) assuming unequal variances for the two sets.

DATA AVAILABILITY: The data that support the findings of this study are available from the corresponding authors upon reasonable request.

AUTHOR CONTRIBUTIONS: The manuscript was written by RWC and HP. Conceptual input was provided by RWC, FAF, AWW, TG, and HLP. Experiments were designed and performed by RWC, FAF, ED, NS and TG.

COMPETING INTERESTS: RWC, TG and HLP have filed a provisional patent covering the use of VHH fusions for targeting GPCRs.

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SUPPORTING INFORMATION

Improved GPCR ligands based on genetically impossible nanobody-peptide fusions.

Peptide	[M+H] _{calc}	[M+H] _{obs}
PTH(1-9)	1127.6	1127.1
PTH(1-10)	1255.6	1255.1
PTH(1-11)	1411.7	1411.2
PTH(1-14)	1796.9	1796.3
PTH(1-34)	4218.2	4217.5
PTH(1-9)-dbco	1554.8	1554.2
PTH(1-10)-dbco	1682.8	1682.2
PTH(1-11)-dbco	1838.9	1839
PTH(1-14)-dbco	2224.1	2223.5
PTH(1-34)-dbco	4645.4	4644.6
PTH(1-11)-PEG3-dbco	2042	2041.4
PTH(1-34)-PEG3-dbco	4848.5	4847.7
G ₃ -PTH(1-14)	1865	1864.2

Supporting Figure 1. Confirmation of peptide identity using mass spectrometry. Peptides were analyzed by LC/MS as described in methods. Calculated masses ([M+H]_{calc}) refers to the monoisotopic mass of a singly protonated species. The masses recorded using mass spectrometry are labeled as [M+H]_{obs}.

Conugate	MW _{calc}	MW _{obs}
VHH _{GFP} -G3-Lys(biotin)AhxLys(azide)	n/a	14080
VHH _{GFP} -PTH(1-9)	15635	15635
VHH _{GFP} -PTH(1-10)	15763	15765
VHH _{GFP} -PTH(1-11)	15919	15920
VHH _{GFP} -PTH(1-14)	16304	16305
VHH _{GFP} -G3-PTH(1-14)	15135	15135
VHH _{GFP} -PTH(1-34)-PEG	18929	18930
VHH _{6E} -G3-Lys(biotin)AhxLys(azide)	n/a	13325
VHH _{6E} -PTH(1-9)	14880	14895
VHH _{6E} -PTH(1-10)	15008	15010
VHH _{6E} -PTH(1-11)	15164	15165
VHH _{6E} -PTH(1-11)-PEG	15367	15375
VHH _{6E} -PTH(1-14)	15549	15550
VHH _{6E} -PTH(1-34)-PEG	18174	18175
VHH _{6E} -G3-PTH(1-14)	14380	14380
VHH _{PTHR} -PTH(1-9)	16505	16505
VHH _{PTHR} -PTH(1-10)	16633	16635
VHH _{PTHR} -PTH(1-11)	16789	16790
VHH _{PTHR} -PTH(1-14)	17174	17175
VHH _{PTHR} -G3-Lys(biotin)AhxLys(azide)	n/a	14950
VHH _{PTHR} -G3-PTH(1-14)	16005	16005

Supporting Figure 2. Confirmation of VHH-peptide conjugate identity using mass spectrometry. VHH-peptide conjugates were analyzed by LC/MS as described in methods. Deconvolution calculations were used to provide the observed values. MW_{calc} refers to the calculated average molecular weight and MW_{obs} refers to the molecular weight recorded by mass spectrometry.

Supporting Figure 3. Assessment of VHH binding to PTHR1 variants expressed on HEK293 cell lines by flow cytometry. Cells dislodged from tissue culture plates using trypsinization were incubated with varied concentrations of VHHs sortagged with AlexaFluor647 on ice for 1 h. Cells were centrifuged, washed, and analyzed by flow cytometry via gating of intact cells based on forward scatter/side scatter profiles. Data points represent median fluorescent intensity values (mean $\pm$ SD). Connecting curves are the result of fitting a sigmoidal dose-response model to the data points. The plateau for maximum labeling using VHH_{PTHR} was estimated based on maximal labeling with other VHHs.

Supporting Figure 4: Assessment of VHH_{6E} and PTH(1-20) binding to PTHR1_{6E} using microscopy. Adherent HEK293 cells expressing human PTHR1 were stained on ice with 300 nM VHH_{6E}-TMR and 30

nM PTH(1-20)-FAM for 30 minutes. Following staining cells were washed and treated with fixative in preparation for image acquisition either immediately after staining (0 m) or following a 15 minute incubation in medium at room temperature (15 m).

Supporting Figure 5. Representative dose-response curves for cAMP induction in HEK293 cell lines. HEK293 cell lines were activated with peptides or conjugates as described in methods. Data points represent mean $\pm$ SD and connecting lines result from the fit of a four-parameter sigmoidal dose-response model. Cell lines stably express (a) PTHR1_{GFP}, (b) PTHR1_{6E}, (c) PTHR1_{YFPΔECD}, or (d-e) human PTHR1.

Supporting Figure 6. Modification of the N-terminus of PTH degrades activity. (a) Schematic comparison of the topology of the two types of conjugates tested. (b) Representative dose-response curves for activation of human PTHR1 by indicated peptides or conjugates run as described in methods. Data points indicate mean ± SD and connecting lines result from the fit of a four-parameter sigmoidal dose-response model. (c) Tabulation of cAMP induction potencies.

Supporting Figure 7. VHH anchoring of PTH fragments prolongs cAMP signaling. Cells were treated with peptides or conjugates at concentrations listed in the legend for each figure to stimulate cAMP responses as described in methods. **(a)** Scheme describing workflow for cAMP kinetics experiments. cAMP responses were recorded every two minutes following addition of peptide and after washout of free peptide. The time needed for medium removal, washing of cells, and resuspension in fresh medium spans approximately 2 minutes. **(b)** Representative plots of the kinetics of cAMP-induced signal production (left) and signal cessation after removal of medium containing from ligand (right) in hPTH1R1 expressing HEK293 cells. Ligands were used at the minimal concentration that stimulated near maximal cAMP responses to minimize effects from non-specific adherence. Data points indicate mean $\pm$ SD from three replicates. Lines connect data points and only serve to guide the eye. The ligand on phase was omitted from panels c-h but each ligand tested induced a similar cAMP response prior to washout (data not shown). **(c-h)** Ligand off phase following stimulation of cells expressing **(c-e)** PTHR1_{6E} or **(f-h)** PTHR1_{YFPAECD}. Data from individual cell lines are separated into separate panels for clarity.

Supporting Figure 8. Variation in binding caused by conjugation of receptor-binding or irrelevant VHHs. HEK293 cells expressing PTHR1 were stained with conjugates indicated in panels a-d at concentrations listed in legends and prepared for analysis by flow cytometry as described in methods. The control staining condition for each panel was staining with VHH_{6E} -biotin-azide used at a concentration of 100 nM. In panel b, VHH_{PTHR} -biotin-azide was used for staining.

Supporting Figure 9. Impact of DBCO conjugation and PEG linker insertion on peptide and conjugate bioactivity. Peptides and VHH-peptide conjugates were assessed for cAMP induction in HEK293 cell lines as described in methods. Data points represent mean ± SD and connecting lines result from the fit of a four-parameter sigmoidal dose-response model. **(a)** Structure of PTH(1-11) fused to either DBCO or PEG₃-DBCO to illustrate connectivity. Atoms corresponding to the PEG linker are highlighted in red. **(b-c)** Representative dose-response curves for stimulation of human PTHR1. **(b)** Insertion of a PEG₃ linker does not enable activation of receptors not bound by VHHs by VHH-PTH(1-11) conjugates. **(c)** Attachment of DBCO or PEG-DBCO to PTH(1-11) does not substantially alter receptor activation properties. **(d)** Tabulation of experimental results for activation of hPTH1R by PTH and conjugates. These data are distinct from those presented in Table 1. **(e)** Representative dose-response curve for stimulation of PTHR1_{6E} by DBCO and PEG-DBCO conjugates of PTH fragments. Insertion of a PEG₃ linker does not substantially alter receptor activation properties. **(f)** Tabulation of experimental results for activation of PTHR1-6E by PTH and conjugates. These data are distinct from those presented in Table 1.

Supporting Figure 10. VHH conjugation does not affect signaling capacity of PTH(1-34). PTH(1-34)-Cys was conjugated to VHH with an intervening PEG₃ linker as described in Figure 2 and Supporting Figure 8. The induction of cAMP responses was assessed in cell lines expressing (a) hPTH1R or (b) PTHR1_{6E}. Representative dose-response curves are shown in which data points indicate mean ± SD and connecting lines result from the fit of a four-parameter sigmoidal dose-response model. Composite results are tabulated below the dose-response curves.

Supporting Figure 11. Measurement of cytoplasmic calcium mobilization by PTHR1 agonists. HEK293 cells were loaded with FURA2 then stimulated with (a) PTH(1-34), (b) VHH_{PTH1R}-PTH(1-11) or (c) VHH_{PTH1R}-PTH(1-14) at time zero as described in the methods section. The colors used to represent each concentration are held consistent in each panel. Data points indicate mean ± SEM from two independent measurements.

b)

Peptide/conjugate	EC ₅₀ (nM ± SD)			<i>n</i>
PTH(1-34)	0.28	±	0.12	3
PTH(1-11)	120.3	±	90.3	3
PTH(1-10)	2466	±	23	3
VHH _{PTHR} -PTH(1-11)	3.2	±	0.9	3
VHH _{6E} -PTH(1-11)	>100 nM			2
VHH _{GFP} -PTH(1-11)	>100 nM			2

Supporting Figure 12. VHH_{PTHR} conjugation potentiates PTH fragment activation of rat PTHR1. HEK293 cells stably expressing rat PTHR1 were stimulated with peptide or conjugate as described in methods. (a) Representative dose-response curve for stimulation of rPTH1 by peptides or VHH-PTH(1-11) conjugates. Data points indicate mean ± SD and connecting lines result from the fit of a four-parameter sigmoidal dose-response model. (b) Tabulation of composite results from rPTH1 cAMP stimulation assays.

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PTHR6E    MGTARIAPGLALLCCPVLSSAYALVDADDVMTKEEQIFLLHRAQAQCEKRLKEVLQRPA 60
Align     MGTARIAPGLALLCCPVLSSAYALVDADDVMTKEEQIFLLHRAQAQCEKRLKEVLQRPA
hPTRH     MGTARIAPGLALLCCPVLSSAYALVDADDVMTKEEQIFLLHRAQAQCEKRLKEVLQRPA 60

PTHR6E 61  SIMESDKGWTQADQEAKELARQISSGKLYPESEEDKEAPTGSRYRGRPCLPEWDHILCWPL 120
Align     SIMESDKGWT A      K      + SGKLYPESEEDKEAPTGSRYRGRPCLPEWDHILCWPL
hPTRH1 61  SIMESDKGWTSASTSGKPRKDKASSGKLYPESEEDKEAPTGSRYRGRPCLPEWDHILCWPL 120

PTHR6E 121  GAPGEVVAVPCPDYIYDFNHKGHAYRRCDRNGSWELVPGHNRTWANYSECVKFLTNETRE 180
Align     GAPGEVVAVPCPDYIYDFNHKGHAYRRCDRNGSWELVPGHNRTWANYSECVKFLTNETRE
hPTRH1 121  GAPGEVVAVPCPDYIYDFNHKGHAYRRCDRNGSWELVPGHNRTWANYSECVKFLTNETRE 180

PTHR6E 181  REVFDRLGMIYTVGYSVSLASLTVAVLILAYFXRLHCTRNYIHMHLFLSFMLRAVSIFVK 240
Align     REVFDRLGMIYTVGYSVSLASLTVAVLILAYF RLHCTRNYIHMHLFLSFMLRAVSIFVK
hPTRH1 181  REVFDRLGMIYTVGYSVSLASLTVAVLILAYFRRLHCTRNYIHMHLFLSFMLRAVSIFVK 240

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Supporting Figure 13. Annotated sequence data for PTHR1_{6E}. The color code for highlighting in the sequence is as follows: Green- sequences flanking exon 2 that are resolved in the crystal structure (PDB: 6FJ3); Cyan-resolved not in crystal structure; Yellow-site of 6E tag incorporation (not resolved in crystal structure. Sequence corresponding to Exon 2 is underlined.

MGTARIAPGLALLCCPVLSSAYALATMVSKEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKICTTGKLPVPWPTLVTTLGYGVQCFAFYPDHMKQH
DFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYSNHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQNTPIGD
GPVLLPDNHLYSYQSKLSKDPNEKRDHMLLEFVTAAGITLGMDELYKSGEVFDRLGMIYTVGYSVSLASLTAVLILAYFRRHLCTRNYIHMHFLFSFMLRAVSIFVKD
AVLYSGATLDEAERLTEEELRAIAQAPPPATAAAGYAGCRVAVTFFLYFLATNYYWILVEGLYLHSLIFMAFFSEKKYLWGFTVFGWGLPAVFVAVWVSVRATLANTG
CWDLSSGNKKWIIQVPILASIVLNFILFINIVRVLATKLRTNAGRCDTRQQYRKLLKSTLVLMPLFGVHYIVFMATPYTEVSGTLWQVQMHYEMLFNSFQGFVVAIYCFC
NGEVQAEIKKSWSRWTLALDFKRKARSGSSSYSGPMVSHTSVTNVGPVGLGLPLSPRLPTATTNGHPQLPGHAKPGTPALETLETTTPAMAAPKDDGFLNGSCSG
LDEEASGPERPPALLQEEWETVM

Supporting Figure 14. Sequence of PTHR1_{YFPΔECD}. Sequence corresponding to hPTHR1 is highlighted in cyan. Sequence corresponding to YFP is highlighted in yellow.

	hPTHr1				hPTHr1 _{opp}				hPTHr1 _{eg}			
	EC ₅₀ (nM ± SD)	Max (normalized ± SD)	n		EC ₅₀ (nM ± SD)	Max (normalized ± SD)	n		EC ₅₀ (nM ± SD)	Max (normalized ± SD)	n	
PTH(1-34)	0.51 ± 0.28	1.00 ± 0.00	8		0.4 ± 0.8	1.00 ± 0.00	7		1.3 ± 1.0	1 ± 0.0	3	
PTH(1-14)	4.3 ± 2.0	1.01 ± 0.07	5		2.3 ± 1.2	1.09 ± 0.03	3		3.4 ± 1.6	1.06 ± 0.12	3	
PTH(1-11)	516 ± 238	1.01 ± 0.09	7		79 ± 45	1.10 ± 0.13	7		94 ± 74	1.09 ± 0.22	3	
PTH(1-10)	3121 ± 1671	1.03 ± 0.08	3		2552 ± 653	1.16 ± 0.15	6		5079 ± 407	1.09 ± 0.07	3	
PTH(1-9)	Inactive at 10,000 nM		3		Inactive at 10,000 nM		4		Inactive at 10,000 nM		3	
VHH _{FMET} -PTH(1-14)	0.075 ± 0.041	0.96 ± 0.17	4		1.7 ± 2.3	1.10 ± 0.10	3		0.2 ± 0.1	0.98 ± 0.19	3	
VHH _{FMET} -PTH(1-11)	5.0 ± 1.6	0.95 ± 0.09	7		0.5 ± 0.1	1.03 ± 0.26	4		4.0 ± 3.2	0.98 ± 0.11	3	
VHH _{FMET} -PTH(1-10)	Inactive at 100 nM		2		ND				ND			
VHH _{FMET} -PTH(1-14)	Inactive at 330 nM		3		32.9 ND 3.5	1.14 ± 0.08	3		0.4 ± 0.2	1.08 ± 0.23	3	
VHH _{FMET} -PTH(1-11)	Inactive at 100 nM		2		Inactive at 100 nM		3		6.9 ± 2.6	1.11 ± 0.18	3	
VHH _{FMET} -PTH(1-10)	ND				Inactive at 100 nM		3		2.8 ± 1.4	0.94 ± 0.04	3	
VHH _{FMET} -PTH(1-9)	ND				ND				Inactive at 100 nM		3	
VHH _{GGP} -PTH(1-14)	Inactive at 100 nM		3		1.8 ± 0.6	1.21 ± 0.17	3		Inactive at 100 nM		2	
VHH _{GGP} -PTH(1-11)	Inactive at 100 nM		3		8.0 ± 3.4	1.10 ± 0.18	6		Inactive at 100 nM		2	
VHH _{GGP} -PTH(1-10)	ND				Inactive at 100 nM		3		ND			
VHH _{GGP} -PTH(1-9)	ND				Inactive at 100 nM		4		ND			

	hPTHr1 _{YFPΔECD}				rPTHr1				hPTHr2			
	EC ₅₀ (nM ± SD)	Max (normalized ± SD)	n		EC ₅₀ (nM ± SD)	Max (normalized ± SD)	n		EC ₅₀ (nM ± SD)	Max (normalized ± SD)	n	
PTH(1-34)	689 ± 301	1.00 ± 0.00	7		0.28 ± 0.12	1.00 ± 0.00	3		1.45 ± 2.42	1 ± 0	4	
PTH(1-14)	1.1 ± 0.9	0.96 ± 0.04	4						924 ± 328	1.14 ± 0.28	3	
PTH(1-11)	246 ± 133	1.02 ± 0.12	10		120.3 ± 90.3	1.02 ± 0.03	3		>100,000 (20% at 100 uM)	0.36 ± 0.32	3	
PTH(1-10)	3841 ± 1604	1.12 ± 0.08	6		2466 ± 23	1.09 ± 0.11	3				4	
PTH(1-9)	Inactive at 10,000 nM		3								3	
VHH _{FMET} -PTH(1-14)	0.9 ± 0.5	0.91 ± 0.15	4		3.2 ± 0.9	1.00 ± 0.13	3		Inactive at 330 nM			
VHH _{FMET} -PTH(1-11)	Inactive at 100 nM		3									
VHH _{FMET} -PTH(1-10)	ND											
VHH _{FMET} -PTH(1-14)	72 ± 15.2	1.11 ± 0.07	3									
VHH _{FMET} -PTH(1-11)	Inactive at 100 nM		3		Inactive at 100 nM		2					
VHH _{FMET} -PTH(1-10)	ND											
VHH _{FMET} -PTH(1-9)	ND											
VHH _{GGP} -PTH(1-14)	0.58 ± 0.29	1.16 ± 0.36	4									
VHH _{GGP} -PTH(1-11)	0.14 ± 0.06	1.19 ± 0.40	7									
VHH _{GGP} -PTH(1-10)	0.46 ± 0.22	1.06 ± 0.35	7		Inactive at 100 nM		2					
VHH _{GGP} -PTH(1-9)	~40% activation at 100 nM	0.38 ± 0.11	5									

Supporting Table 1. Complete tabulation of cAMP induction assays. Data are identical to those presented in Table 1 and Supporting Figure 12. Maximal responses (“Max”) were normalized relative to the top plateau value induced by saturating values of PTH(1-34) for all receptors besides hPTHr1_{YFPΔECD}, for which PTH(1-11) was used.

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