JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/jacsau
Article

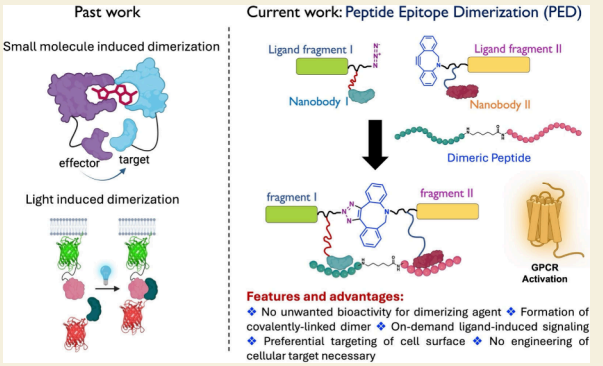
Chemically Induced Dimerization via Nanobody Binding Facilitates in Situ Ligand Assembly and On-Demand GPCR Activation

Shubhra Jyoti Saha and Ross W. Cheloha*

Cite This: JACS Au 2024, 4, 4780−4789
Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Methods that enable the on-demand synthesis of biologically active molecules offer the potential for a high degree of control over the timing and context of target activation; however, such approaches often require extensive engineering to implement. Tools to restrict the localization of assembly also remain limited. Here we present a new approach for stimulus-induced ligand assembly that helps to address these challenges. This methodology relies on the high affinity and specificity recognition exhibited by antibody fragments (nanobodies, Nbs). By using Nbs that recognize short peptide epitopes to create semisynthetic conjugates, we develop a bioengineering platform termed peptide epitope dimerization (PED) in which the addition of heterodimeric peptide composed of two Nb epitopes stimulates the proximity-induced synthesis of a functional ligand for the parathyroid hormone receptor-1, a G protein-coupled receptor. We further demonstrate that high efficiency assembly can be achieved on the cell surface via Nb-based delivery of template. This approach opens the door for the on-demand generation of bioactive receptor ligands preferentially at a desired biological niche.



KEYWORDS: *click chemistry, peptide, nanobody, templated synthesis, GPCR*

INTRODUCTION

One approach for the design of compounds that act with spatiotemporal and target specificity centers on the use of building blocks that can react to form a bioactive product upon exposure to a certain stimulus that occurs only at the desired site of action. Most established examples of such strategies center on transformations upon prodrugs executed by enzymes or cells enriched at the same anatomical location as the target of interest.[1] Such approaches are often limited to the activity of naturally occurring enzymes, which sometimes lack anatomical specificity. Alternative approaches, such as splitting biomolecules into nonfunctional subunits that combine only when bound to target(s) of interest, offer promising possibilities for enhancing specificity.[2] Small molecule-mediated chemically induced dimerization (CID)[3] is frequently achieved through the rapamycin-induced assembly of fusion proteins consisting of FK506-binding protein (FKBP) and FKBP−rapamycin binding domain (FRB).[4] Other commonly used systems induce dimerization through the addition of abscisic acid (ABA)[5] or an analogue of gibberellic acid.[6] Biological engineering with light-sensitive building blocks has also been shown to facilitate illumination-induced dimerization and downstream biological effects.[7] Herein, we describe a novel alternative for stimuli-inducible assembly that relies on a peptide trigger of dimerization (peptide epitope dimerization, PED) that results in the production of a bioactive compound. This approach offers a distinct set of features that are useful for control of biological activity at the cell surface.

We apply this approach to target a member of the G protein-coupled receptor (GPCR) superfamily, parathyroid hormone receptor-1 (PTHR1).[8] GPCRs are cell surface membrane proteins that mediate diverse physiological processes and are leading targets for drug development.[9] GPCRs and their endogenous ligands are known to exhibit promiscuity, with one receptor binding many natural ligands and one ligand binding to many receptors.[10] Furthermore, studies have revealed that many GPCRs induce signaling through multiple distinct G protein isoforms or arrestins, which is often dependent on the identity of the ligand bound to the receptor, in a process known as ligand biased agonism.[11] We hypothesized that the *in situ* synthesis of a GPCR ligand could provide a new approach to implement receptor- and pathway-specific activation of GPCRs.

We describe the development and application of a peptide template-induced dimerization strategy to facilitate *in situ*

Received: August 6, 2024
Revised: November 16, 2024
Accepted: November 18, 2024
Published: November 25, 2024

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

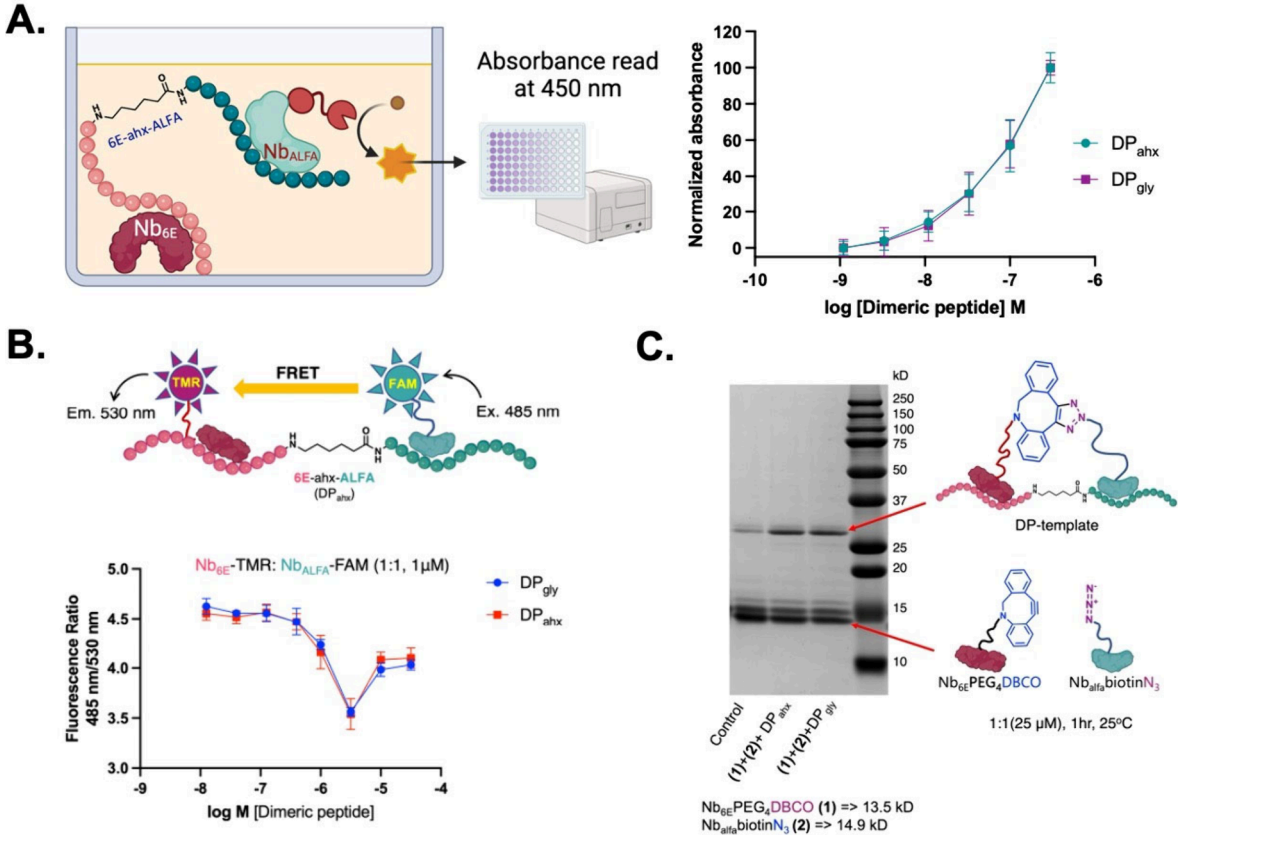


Figure 1. Assessment of DP-induced dimerization of Nbs. (A) (Left) Schematic of a sandwich ELISA to confirm simultaneous binding of DP to Nb_{6E} and Nb_{ALFA}. (Right) Concentration dependent increase in the ELISA signal upon treatment with varying concentrations of dimeric peptides (DP_{gly} and DP_{ahx}). Data points correspond to mean ± SD from technical replicates. Independent biological replicates are shown in Supporting Figure S2A. (B) Dimeric peptides (DP_{gly} or DP_{ahx}) induce FRET between fluorophore-labeled Nbs. Measurements of the fluorescence intensity were recorded with equal concentrations (1 μM) of Nb_{6E}-TMR and Nb_{ALFA}-FAM in the presence of varying concentrations of DPs. Data points correspond to mean ± SD from technical replicates performed in a single representative experiment. Measurements were performed immediately after the addition of DP. Independent biological replicates are shown in Supporting Figure S2B. (C) DP-induced reactivity between Nbs labeled with azide and alkyne handles. Analysis of cross-linking was performed using SDS-PAGE (15% acrylamide). After 1 h, reactions were quenched with excess of GGG-Lys(N_3) peptide (500 μM) to react with any residual Nb_{6E}-PEG_4-DBCO and prevent further cross-linking. The control condition (left lane) lacks DP. The identity of cross-linked product was confirmed using mass spectrometry (Supporting Figure S3A). Possibility of spontaneous heterodimerization of unlabeled Nbs was investigated using SDS-PAGE (15% acrylamide) and mass spectrometry (Supporting Figure S3B and C). Experimental details are found in the Methods.

production of bioactive ligand for PTHR1 from weakly active and inactive building blocks.[12,13] Previously published work from our group described the generation Nb-PTH conjugates were highly active at PTHR1[13] and exhibited pathway specific signaling properties,[14] but were not subject to control using an outside trigger, unlike the approach described in this study. By enforcing the proximity between azide- or alkyne-linked ligand fragments (PTH1−11 and PTH12−34), we catalyze the *in situ* synthesis of an analogue of a prototypical peptide agonist of PTHR1 (PTH1−34). In this context, dimerization, assembly, and biological activity are driven by the high affinity recognition of short peptide epitopes by single domain antibodies (or nanobodies, Nbs).[15,16] Dimeric peptides (DPs), comprising a fusion of peptide epitopes recognized by two different Nbs, were developed as templates for PED. Past work has shown that dimerization of PTHR1 ligands provides highly active ligands, offering support for the feasibility of this approach.[17] Here we describe the development of this approach and its application to rapid, on-demand assembly conjugates that stimulate GPCR signaling responses.

RESULTS AND DISCUSSION

We synthesized heterodimeric peptides designed to simultaneously bind to two distinct Nb epitopes and induce proximity. The dimeric peptides consisted of a 6E peptide (epitope tag bound by Nb_{6E})[15,18] connected to a ALFA peptide (bound by Nb_{ALFA})[19] with a glycine (DP_{gly}) or 6-aminohexanoic acid (DP_{ahx}) linker (Supporting Table S1). The comparison of either glycine or ahx interposed between epitope tags allowed us to evaluate the role of the linker length as a variable. First, we tested whether DP_{gly} (or DP_{ahx}) could simultaneously bind to both epitope tag-binding nanobodies (Nb_{6E} and Nb_{ALFA}). A sandwich enzyme-linked in situ assay (ELISA) was performed by using a format that specifically measured the binding of DP to both Nb_{6E} and Nb_{ALFA} (Figure 1A). Nb_{6E} was immobilized and varying concentrations of DP_{gly} (or DP_{ahx}) were subsequently added. After washing steps, a second nanobody bearing a biotin (Nb_{ALFA}-biotin-azide, or "Nb_{ALFA}-BA"; Supporting Table S2 and Supporting Figure S1), produced by enzymatic labeling[20] with Sortase 5M, was added. Plate bound Nb_{ALFA}-BA was detected and quantified by using streptavidin-conjugated horseradish peroxidase (SA-HRP) in a

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

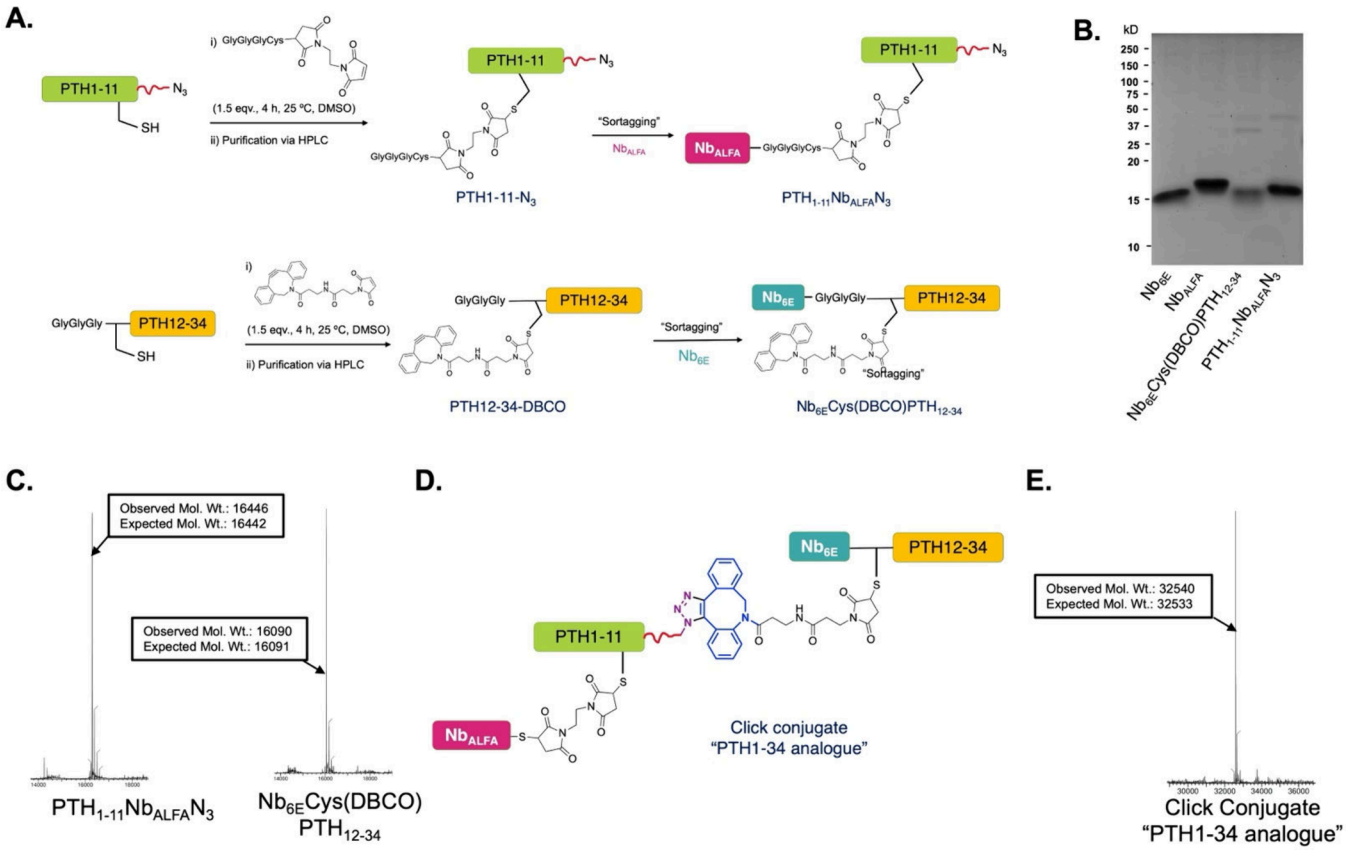


Figure 2. Synthesis and characterization of Nb-PTH conjugates for DP-induced assembly. (A) Scheme for synthesis of clickable Nb-peptide conjugates. Mass spectrometry characterization of intermediates is found in Supporting Table S1. Structures of peptide building blocks and Nb-peptide conjugates are found in Supporting Figure S1. Conditions for sortagging are described in Methods. (B) SDS-PAGE analysis of Nbs and conjugates described in panel (A). (C) Mass spectrometry characterization of substrates from panel (B). (D) Assembly of PTH1−34 analogue from Nb-PTH building blocks. (E) Mass spectrometry characterization of the fully assembled product shown in panel (D). A mass spectrum showing a wider mass range is included in Supporting File 1.

standard colorimetric assay. The data indicated a dose-dependent increase in signal for increasing concentrations of DP_{gly} (or DP_{ahx}) (Figure 1A and Supporting Figure S2A). Next, we performed a Förster resonance energy transfer (FRET) assay to assess whether DP binding brings the tag-binding Nbs into the proximity, a requirement for templated click reactions. FRET only occurs when the donor and acceptor moieties reside within proximity (typically 1−10 nm).[21] Using sortagging, we labeled Nb_{6E} and Nb_{ALFA} with suitable acceptor (Tetramethylrhodamine (TMR)) and donor (5(6)-Carboxyfluorescein (FAM)) fluorophores, respectively (Supporting Table S2). Nb_{ALFA}-FAM was excited at 485 nm and emission of TMR labeled Nb_{6E} was recorded at 530 nm. Results showed a decrease in the ratio of fluorescence measured at different wavelengths (485/530 nm) with increasing concentrations of the DP template, indicating FRET mediated by binding of Nbs to DP (Figure 1B and Supporting Figure S2B). We noted a decrease in FRET measured at the two highest concentrations of DP (Supporting Figure S2B), likely related to a "hook effect", frequently observed upon application of an excess of heterobivalent compounds.[22]

We next sought to test whether the peptide-induced proximity could be used to template a reaction between suitably functionalized Nb-based fragments. Previous reports have demonstrated successful use of oxime ligation,[23] hydrazone formation,[24] thioesterification,[24] amide bond synthesis,[24] and cysteine-maleimide linkage[25] to produce desired conjugates from fragments upon exposure to a biomolecule or cell that templates the reaction. Molecular recognition events have also been used template cycloaddition or "click reactions" between reactive fragments bearing azide- and alkyne-bearing functionalities, including applications with antibody targets, nucleic acids (DNA), and ribosomes.[26−28]

We evaluated the feasibility of a DP template-induced click reaction between Nb conjugates bearing complementary click handles (Figure 1C and Supporting Figure S3A). We opted to use a strained alkyne (dibenzylcyclooctyne, DBCO), which is known to engage in a metal-free cycloaddition[29] reaction with azides, albeit at a relatively slow rate (~1 $M^{-1}S^{1-}$) in the absence of proximity enforcing conditions.[30] We hypothesized that the DBCO-azide click reaction would exhibit relatively slow background reactivity that could be accelerated through enforced proximity. Once again, we used sortagging to attach either an alkyne (G_3DBCO) or azide (G_3Lys(biotin)Lys-(azide)) moiety (Supporting Figure S1) to individual Nbs. Analysis of this reaction by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that a 1 h reaction between Nbs bearing complementary click handles produces a high molecular weight band that corresponds to the heterodimeric product (Figure 1C and Supporting Figure S3A). The intensity of the band corresponding with the product of the click reaction was more intense in the presence of DP (containing either a Gly or

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

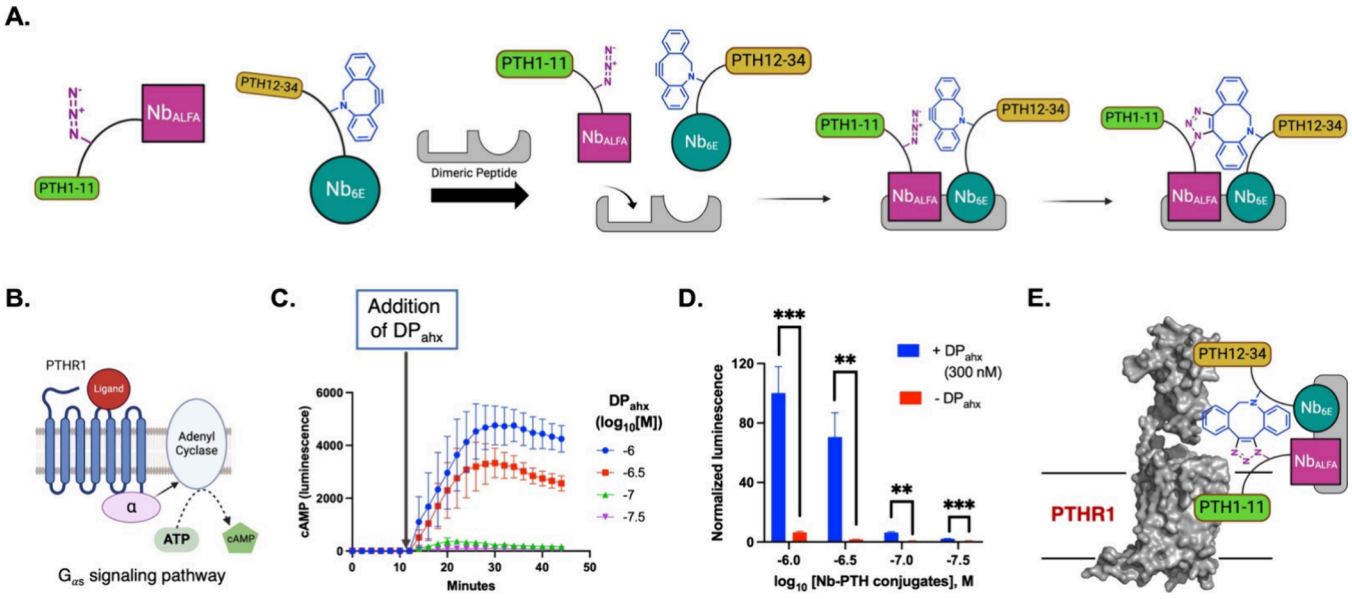


Figure 3. Template-induced dimerization mediates activation of PTHR1. (A) Schematic of DP-induced click reaction and assembly of an analogue of PTH1−34. (B) Simplified schematic of PTHR1 activation induced GαS signaling. (C) Kinetic data showing cAMP responses in cells expressing PTHR1. Cells were incubated in a 96-well plate with an equimolar mixture (1 μM) of $PTH_{1-11}Nb_{ALFA}N_3$ and $Nb_{6E}Cys(DBCO)PTH_{12-34}$ and cAMP responses were measured. After 12 min of incubation, varying concentrations of DP_{ahx} were added (arrow) to wells preincubated with Nb conjugates and cAMP responses were measured for an additional 30 m. Error bars correspond to standard deviation of luminescence measurements recorded in technical replicates in a representative experiment. Independent biological replicates are shown in Supporting Figure S6. A comparative analysis of cAMP responses between PTH1−34 and Nb-PTH conjugates is shown in Supporting Figure S7. (D) Induction of cAMP responses in cells expressing PTHR1 by Nb-PTH conjugates in the presence or absence of DP_{ahx} (300 nM). Varying concentrations of $PTH_{1-11}Nb_{ALFA}N_3$ and $Nb_{6E}Cys(DBCO)PTH_{12-34}$ were added to cells in either the presence or absence of dimeric peptide (300 nM). All responses were normalized to the maximum luminescence observed for Nb-PTH conjugates (1 μM). Error bars correspond to technical replicates in a representative experiment. Statistical significance (p-value) was assessed by unpaired nonparametric *t* test (**$p < 0.01$; ***$p < 0.001$). Independent biological replicates are shown in Supporting Figure S8. (E) Simplified schematic of DP facilitating PTHR1 activation via a templated reaction between PTH-Nb fragments.

Ahx linker) than in their absence, indicating that enforced proximity accelerates azide−alkyne cycloaddition chemistry. Mass spectrometry confirmed that the high molecular weight band corresponded to a heterodimeric product (Supporting Figure S3A). This analysis also revealed the presence of proteins of a slightly lower molecular weight than the azide- or DBCO-labeled Nb starting materials, corresponding to hydrolysis side products commonly produced by sortagging reactions, or other unknown contaminants (see Supporting Information for more details). A control experiment involving coincubation of Nb_{6E} and Nb_{ALFA} (without appended click handles) demonstrated very low levels of dimerization, indicating that dimerized product results from azide−alkyne chemistry and not spontaneous cross-linking (Supporting Figure S3B and C). We confirmed that neither different length linkers (Gly vs Ahx) within DPs or reversing the pairing of the click chemistry handle (azide vs alkyne) attached to Nbs had substantial impacts on cross-linking kinetics (Supporting Figure S4A−C). These data support the conclusion that DP accelerates cross-linking, with no major differences observed between DP_{Gly} and DP_{Ahx}.

Subsequently, we sought to test whether the *in situ* click assembly could be used to induce receptor activation. Toward this goal, we used weakly active or inactive fragments of a PTHR1 agonist peptide (PTH1−34; PTH1−11 + PTH12−34).[13] We used an analogue of PTH1−11 (referred to as PTH1−11 hereafter) known to have improved bioactivity relative to the wild type sequence (see Supporting Table S1 for peptide sequence and Supporting Information Figure S1 for structure). We linked these PTH ligand fragments to Nbs (Nb_{6E} or Nb_{ALFA}) and click chemistry handles (azide and DBCO) to facilitate DP-induced assembly (Figure 2A and Supporting Table S2). We hypothesized that DP_{gly} (or DP_{ahx}) would behave as a template that would promote proximity and an accelerated click reaction to synthesize an analogue of PTH1−34, with analogy to experiments performed with Nbs labeled with click handles (Figure 1C). We used solid phase peptide synthesis to prepare the PTH fragment peptide PTH1−11-CysAhxLys(azide) to be applied with Gly_3CysPTH12−34 (see Supporting Figure S1 for structures). The introduction of a triglycine (Gly_3 or G_3) motif, either through conventional solid phase peptide synthesis (for PTH12−34 building blocks), or through introduction of a pendant G_3 using Cys-maleimide chemistry (for PTH1−11 building blocks, described below), enables the use of sortase-mediated conjugation chemistry for linkage with Nbs.[20] The Cys introduced into the PTH12−34 fragment enables the introduction of a DBCO moiety using a commercially available maleimide conjugate.

The approach used to enable sortagging with the PTH1−11 fragment merits an additional description (Figure 2A). We carefully avoided any N-terminal modification of the PTH1−11 fragment because of the known detrimental impact such modifications for PTHR1 agonist activity.[31] We therefore developed a new variation for linker chemistry based on the use of a maleimide-functionalized triglycine moiety (Supporting Figure S5) for sortase 5M-mediated conjugation using the C-terminus of PTH1−11. Briefly, a cysteine-maleimide

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

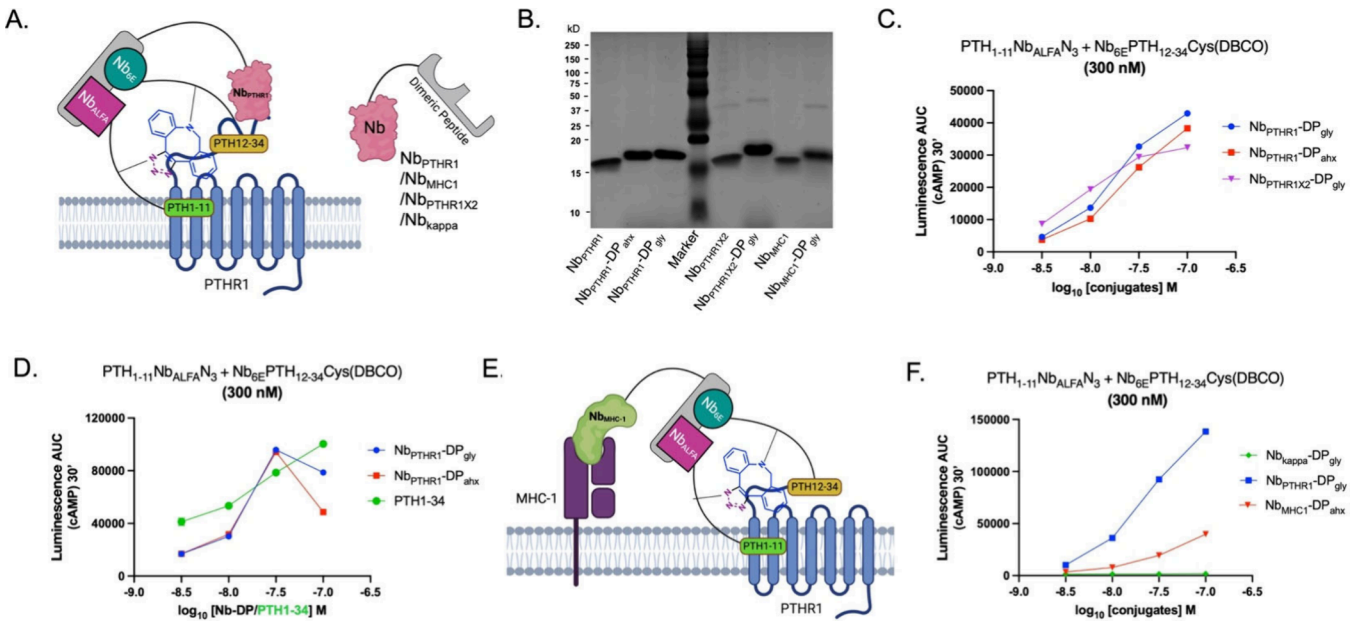


Figure 4. Nb-directed delivery of dimeric peptide template potentiates receptor activation. (A) Schematic of Nb_{PTHR1}-DP conjugates templating ligand assembly at PTHR1. (B) Assessment of Nb-DP conjugate purity using SDS-PAGE. Conjugate identity was confirmed using mass spectrometry (see Supporting Table S2). (C) Quantitation of cAMP responses as a function of Nb-DP concentrations in the presence of 300 nM Nb-PTH conjugates. Data points correspond to mean ± SD from technical replicates in a representative experiment. Independent biological replicates are found in Supporting Figure S10. (D) Comparison of cAMP responses induced by Nb-DP conjugates and the agonist peptide PTH1−34. Nb-PTH conjugates were not added to the wells treated with PTH1−34. Data points correspond to mean ± SD from technical replicates in a representative experiment. (E) Schematic of $Nb_{MHC\text{-}I}$-DP_{gly} conjugates templating ligand assembly, resulting in targeting of PTHR1. (F) Quantitation of cAMP responses as a function of Nb-DP concentrations in the presence 300 nM of Nb-PTH conjugates. Data points correspond to the mean ± SD from technical replicates in a single representative experiment. Independent biological replicates can be found in Supporting Figure S15. For all data panels, error bars are present but too small to visualize.

reaction was used to attach a triglycine motif to the PTH1−11-CysAhxLys(azide). This approach allowed us to avoid the use of the C-terminal azide for Nb conjugation and retain it for proximity-induced click chemistry. The synthesis of Nb-PTH conjugates comprised of PTH1−11 and PTH12−34 building blocks and click chemistry handles (Supporting Figure S1) proceeded smoothly under standard sortagging conditions, although we did observe a small amount of high molecular weight byproducts of unknown identity. A pair of Nb-PTH conjugates ($PTH_{1-11}Nb_{ALFA}N_3$ and $Nb_{6E}Cys(DBCO)$-PTH_{12-34}) were analyzed using SDS-PAGE (Figure 2B) and mass spectrometry (Figure 2C) to confirm their identity. Incubation of equimolar amounts of $PTH_{1-11}Nb_{ALFA}N_3$ with $Nb_{6E}Cys(DBCO)PTH_{12-34}$ for 3 h at 12 °C yielded a product which is essentially a mimic of the full-length peptide PTH1−34 and for clarity we have termed this click conjugate as "PTH1−34 analogue" (Figure 2D). Mass spectra suggested the consistency of the calculated and observed masses of the click product between two reacting building blocks (Figure 2E).

We tested the capacity of mixtures of Nb-PTH conjugates (such as $PTH_{1-11}Nb_{ALFA}N_3$ + $Nb_{6E}Cys(DBCO)PTH_{12-34}$) to activate PTHR1 in the presence or absence of DP (Figure 3A). A potential proximity-driven *in situ* click reaction between two Nb-PTH conjugates was made feasible by the presence of two nanobody-binding epitopes on the DP (Figure 2E). Now, we assessed PTHR1 activation caused by the *in situ* generated "PTH1−34 analogue". We measured the production of a second messenger molecule (cyclic adenosine monophosphate, cAMP) induced by activation of the PTHR1-associated GαS signaling pathway (Figure 3B) using a transfected HEK293 cell line stably expressing PTHR1 and a luciferase biosensor, that is activated by cAMP production.[32] Only weak or negligible activation of PTHR1 was observed upon a brief addition of $PTH_{1-11}Nb_{ALFA}N_3$ + $Nb_{6E}Cys(DBCO)PTH_{12-34}$ in the absence of the DP template (Figure 3C), indicating that untemplated assembly of PTH fragments was too slow to enable *in situ* activation, at least over the time frames (∼30 m) tested. Addition of DP dramatically potentiated the signaling of mixtures of $PTH_{1-11}Nb_{ALFA}N_3$ + $Nb_{6E}Cys(DBCO)PTH_{12-34}$, suggesting that Nb-mediated proximity promotes assembly of an active conjugate from the inactive Nb-PTH conjugates (Figure 3C and Supporting Figure S6). Overnight preincubation ("premix") of $PTH_{1-11}Nb_{ALFA}N_3$ and $Nb_{6E}Cys(DBCO)$-PTH_{12-34}, to allow sufficient time for an untemplated click reaction to occur in the absence of DP, provided a product (PTH1−34 analogue, Figure 2D) that induced comparable but somewhat weaker cAMP responses than those induced by saturating concentrations of a prototypical PTHR1 agonist peptide, PTH1−34 (Supporting Figure S7A and B). The cAMP responses induced by the mixture of DP, $PTH_{1-11}Nb_{ALFA}N_3$ + $Nb_{6E}Cys(DBCO)PTH_{12-34}$ were comparable to but somewhat weaker than those induced by the "premix" mixture described above (Supporting Figure S7B).

We also assessed DP-induced receptor activation using an alternative assay format. A single concentration (300 nM) of the dimeric peptide (DP_{ahx}) was added to wells containing various concentrations of Nb-PTH conjugates. This assay configuration also showed DP-potentiated activation of PTHR1, with the most striking responses observed upon coadministration of DP with Nb-PTH conjugates at a concentration of 1000 or 330 nM (Figure 3D and Supporting Figure S8). Altogether, these observations suggest that the

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

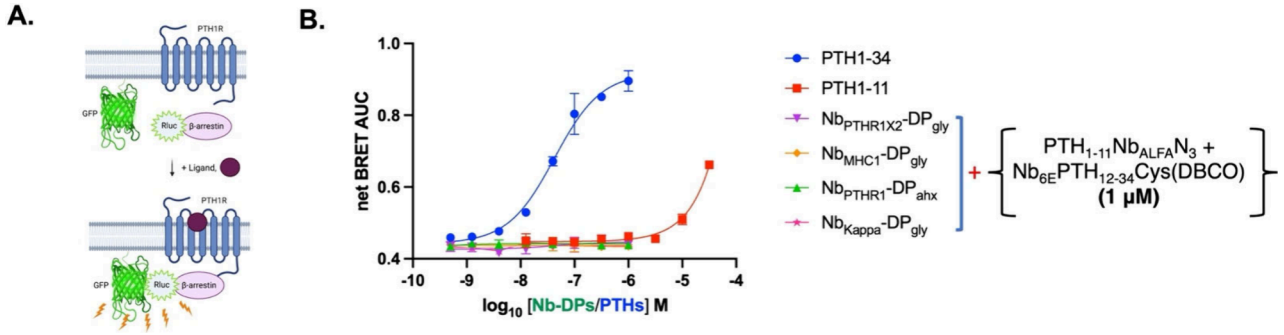


Figure 5. Assessment β-arrestin-2 recruitment to PTHR1 induced by peptide ligands or Nb-PTH conjugates in the presence of Nb-DP. (A) Schematic depicting the mechanism of measurement of PTHR1-mediated β-arrestin recruitment. (B) Dose−response assessment of β-arrestin recruitment induced by peptides or conjugates. Experiments were performed as described in Supporting Methods. The peptide agonist PTH1−11 (see structure in Supporting Figure S1) was included for comparison. Data points correspond to mean ± SD from technical replicates in a representative experiment. Independent biological replicates can be found in Supporting Figure S16.

dimeric peptide acts as a template and brings the Nb-PTH conjugates into proximity to facilitate *in situ* production of a biologically active analogue of PTH1−34, which results in the activation of PTHR1 (Figure 3E). We performed a set of control experiments using a derivative of $Nb_{6E}CysPTH_{12-34}$ in which Cys is functionalized with TMR instead of DBCO and cannot engage in azide−alkyne cross-linking chemistry. The use of $Nb_{6E}Cys(TMR)PTH_{12-34}$ in place of $Nb_{6E}Cys(DBCO)$-PTH_{12-34} results in lower levels of DP-induced activation of PTHR1, suggesting that in situ click chemistry is an important aspect of this approach (Supporting Figure S9).

In the approach described above, DP-induced assembly of Nb-PTH conjugates serves to generate the assembled product in bulk solution, which must still diffuse to reach its target on the cell surface. Such products that fail to reach the cell surface will not effectively activate PTHR1. We hypothesized that tethering the DP template to the cell surface might enable the synthesis of a bioactive conjugate nearer to its site of action (Figure 4A). We conjugated DPs to nanobodies that bind either to PTHR1 ($Nb_{PTHR1,}$ Nb_{PTHRx2}),[14] MHC-I (Nb_{MHC-1}, expressed at high levels on HEK293),[33,34] or to a target not expressed on the surface of HEK293 cells (Nb_{Kappa}, binds to mouse Igκ)[35] (Figure 4A). SDS-PAGE analysis confirmed acceptable purity for the DP-conjugated Nbs (Figure 4B) and mass spectrometry confirmed their identity (Supporting Table S2). As observed for Nb-PTH conjugates, low abundance, high molecular weight bands were observed for specific Nb-DP conjugates in SDS-PAGE analysis.

The purified Nb-DP constructs were assessed for their ability to facilitate receptor activation using the same Nb-PTH conjugates ($PTH_{1-11}Nb_{ALFA}N_3$ and $Nb_{6E}Cys(DBCO)$-PTH_{12-34}) as described above. We observed robust receptor activation with Nb-PTH conjugates when mixed with Nb_{PTHR1}-DP_{ahx}, Nb_{PTHR1}-DP_{gly}, or $Nb_{PTHR1\times2}$-DP_{gly} at Nb-DP conjugate concentrations as low as 10 nM (Figure 4C and Supporting Figure S10). Kinetic evaluation of receptor activation showed that Nb_{PTHR1}-DP_{ahx} mediated rapid activation in a concentration-dependent manner (Supporting Figure S11A). Nb_{PTHR1}-DP_{ahx} exhibited performance superior to that of DP_{ahx} when mixed with Nb-PTH conjugates at a modest concentration (100 nM, Supporting Figure S11B), demonstrating the importance of template delivery to the site of action. Unlike observations with unconjugated DP-mediated activation of PTHR1 (Supporting Figure S6B), receptor activation with Nb_{PTHR1}-DP was comparable to that seen with the prototype peptide PTH1−34 (Figure 4D), further demonstrating the benefit of targeted delivery of the DP template. Interestingly, we observed a diminished cAMP response at the highest concentrations of some of the Nb-DP conjugates tested (Figure 4D and Supporting Figure S11B), perhaps resulting from a "hook effect", as observed frequently with supersaturating concentrations of multivalent conjugates.

To assess whether the assembly mechanism depicted above is accurate, we performed several controls. An analogue of DP in which the sequences of 6E and ALFA peptide are scrambled ($DP_{scrambled}$) was synthesized (Supporting Table S2). We compared $DP_{scrambled}$ to other template peptides for its capacity to induce the activation of PTHR1 (Supporting Figure S11C). Unlike DP_{ahx} and Nb_{PTHR1}-DP_{gly}, $DP_{scrambled}$ did not potentiate the activation of PTHR1 by the Nb-PTH conjugates. We also tested DP and related conjugates for bioactivity or toxicity on HEK293 cells. As expected, DP and Nb-DP conjugates did not induce PTHR1-mediated cAMP responses in the absence of Nb-PTH conjugates (Supporting Figure 12). DP and Nb-DP conjugates were also devoid of any toxicity in cell viability assays (Supporting Figure 13). These findings support the assembly mechanism described above and provide evidence that the templating peptide and related conjugates are biologically inert.

We also tested whether high potency activation requires direct delivery of the DP template to the targeted receptor (PTHR1), or if simply delivering DP to the cell surface (Figure 4E) would suffice. To do so we evaluated Nb_{MHC-1}-DP_{gly} in analogous experiments. In signaling assays, Nb_{MHC-I}-DP_{gly} facilitates activation of receptor substantially more effectively than Nb_{Kappa}-DP_{Gly} (a negative control), although it is less effective than Nb_{PTHR1}- or $Nb_{PTHR1\times2}$-DPs (Figure 4F and Supporting Figure S14). We also performed a variation of this approach in which DP was delivered to the cell surface through an indirect tethering mechanism involving a primary mouse IgG antibody that binds to MHC-I, and a secondary nanobody-DP conjugate (Nb_{Kappa}-DP_{Gly}).[34] In this context, Nb_{Kappa}-DP_{Gly} facilitates low levels of receptor activation when added with a monoclonal antibody that binds MHC-I but not a negative control antibody (Supporting Figure S15A,B). These findings suggest that delivering a DP template to the surface of a targeted cell type can enable ligand assembly and subsequent receptor activation. This approach opens the path toward a logic-gated assembly approach in which the delivery of a

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

template to a cell type or tissue of choice, through attachment to a relevant antibody, could enable spatial restriction on the site of ligand assembly and biological activation.

Stimulation of PTHR1 by PTH1−34 triggers signaling through pathways besides $G\alpha_S$-cAMP, one of which involves the recruitment β-arrestins to cell surface receptor.[8] Certain ligands are reported to preferentially stimulate signaling through one receptor-coupled pathway, but not others, in a paradigm known as "biased agonism".[11] We thus tested whether our *in situ* ligand assembly strategy provided biased agonists. To assess ligand-induced recruitment of β-arrestin 2, we employed a bioluminescence resonance energy transfer (BRET) biosensor-based assay (Figure 5A).[36] An elevated BRET ratio indicates ligand-induced arrestin translocation to the plasma membrane. We evaluated Nb-PTH conjugates administered with Nb-DP templates for their ability to induce β-arrestin 2 translocation. Unexpectedly, we found that the Nb-DP template-induced *in situ* click assembly did not induce arrestin recruitment, whereas peptides PTH1−34 and PTH1−11 individually did (Figure 5B and Supporting Figure S16). While it is possible that assembled Nb-PTH complexes could induce arrestin recruitment at higher concentrations (as was observed for the weak agonist PTH1−11), these studies were not technically feasible, because Nb-PTH conjugates could not be produced in sufficiently concentrated preparations. Full sized proteins (such as the Nbs used to make conjugates) require the retention of a proper fold for function. Concentration of proteins beyond a certain threshold leads to irreversible aggregation and unfolding, which leads to precipitation and loss of biological activity. This issue places an upper bound on the concentrations of preparations that can be attainable for protein-based conjugates. In any case, this finding highlights the possibility of leveraging *in situ* assembly to induce pathway-specific signaling responses, as previously observed for other Nb-ligand conjugates.[37−39]

Previous reports have demonstrated proximity-directed, *in situ* synthesis of inhibitors and ligands for protein receptors and enzymes.[25,26,40−44] By linking reactive functional groups to fragments that individually target a biomolecule of interest with low affinity, simultaneous binding can catalyze the synthesis of a high affinity ligand from fragments. An alternative method for *in situ* formation of bioactive compounds relies on the localized expression of enzymes that can catalyze chemical transformations that either unmask or synthesize biologically active compounds. A distinct approach relies on the transgenic expression of engineered proteins, which upon binding to designed bifunctional molecules, are brought into proximity and exhibit some desirable biological activity, such as targeted killing of tumor cells.[45,46] The PED platform disclosed here differs from some of these previously described approaches in that proximity and reactivity are stimulated by an externally administered reagent (dimeric peptide). Our platform has parallels with rapamycin-mediated chemically induced dimerization;[45,47] however, the templating peptide used in this approach is likely to be devoid of biological action (Supporting Figure S12), unlike rapamycin. Although our method relies on administration of multiple reagents (three separate components), it offers a higher degree of control than alternatives dependent on naturally occurring enzymes or targets. Simplified assembly mechanisms that rely on fewer components may be beneficial in complex biological environments. Future studies will explore alternative approaches to control bioactivity with two inputs instead of three. In contrast to examples in which engineered proteins are expressed in transgenic cells or organisms, our method does not require genetic engineering of targeted proteins.

We have demonstrated the application of our PED approach in a cellular context to induce the activation of a cell surface receptor. One promising observation was that template-induced ligand assembly and corresponding receptor activation was highly efficient when template peptide was linked to a Nb that delivered it to its site of action (Figure 4). Also encouraging is the observation that Nb-DP conjugates that do not target the receptor of interest but instead deliver DP template to the cell surface through binding to coexpressed proteins can still enable target activation. This finding suggests that the delivery of an assembly template to the cells or tissues of choice may enable spatially restricted assembly of biologically active compounds.

Interestingly, *in situ* ligand assembly provided agonists that selectively signal through the $G\alpha s$/cAMP pathway, as evidenced by the lack of β-arrestin 2 recruitment observed upon *in situ* assembly (Figure 5). The current paradigm of how certain ligands display biased agonism involves stabilization of distinct receptor conformations that favor the recruitment of one intracellular signaling component over another. Although PTH1−34 stimulates both $G\alpha s$ activation and β-arrestin recruitment,[48] an analogue of PTH1−34 formed from Nb-conjugated PTH1−11 and PTH12−34 fragments brought together by a dimerizing template only induces $G\alpha s$ pathway signaling. These differences are likely related to the attachment of Nbs to the PTH building blocks, as past work has shown that other Nb-PTH conjugates are also biased toward $G\alpha s$ activation.[37] How the linkage of Nbs to PTH fragments alters the mode of ligand engagement with receptor and how this translates to pathway selective signaling remains to be elucidated. One intriguing possibility is that the appendage of a globular protein (such as a Nb) to the ligand restricts ligand−receptor interactions in a way that precludes a conformational change in receptor required for β-arrestin recruitment. There is a substantial interest in understanding whether alterations in the structure of GPCR ligands that lead to pathway-selective signaling, can be used to induce favorable responses while avoiding side-effects that result from secondary receptor coupling pathways.[49,50] Our findings suggest a novel avenue for identifying biased agonists.

CONCLUSION

The modular and programmable nature of our strategy suggests the possibility of engineering similar methods for the targeted synthesis of a diverse range of biologically active compounds at their site of action *in vivo*. While this approach was used for the synthesis of a GPCR agonist, alternative configurations could be used to generate GPCR antagonists, ligands for other cell surface receptors, or cytotoxic compounds or enzymes.[51] One notable characteristic of this platform is that the components involved in DP-induced assembly are cell impermeable. This feature should enable the preferential targeting of biological recognition and signaling events that occur on the cell surface while avoiding the complications and side-effects that can accompany cell-permeable probes. In conclusion, peptide epitope dimerization (PED) offers an adaptable, efficient, and robust platform that is likely to find use in a variety of biological and therapeutic contexts.

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

EXPERIMENTAL SECTION

Expression and Purification of Nbs from *E. coli*

Nbs (Nb_{6E}, Nb_{ALFA}, Nb_{PTHR1}, $Nb_{PTHR1\times2}$, $Nb_{MHC\text{-}I}$, Nb_{Kappa}) engineered to express near their C-terminus a sortase-recognition motif (LPETG) were produced using recombinant expression in *E. coli* as previously described.[52] Experimental details are provided in the Supporting Methods.

Synthesis, Derivatization, and Characterization of Peptides

All peptides were synthesized using solid-phase synthesis with Fmoc protection of the amino acid backbone, as previously described.[53] Peptide reagents were purified using HPLC and characterized by mass spectrometry (Supporting Table S1). Additional details are provided in the Supporting Methods. Mass spectra and HPLC chromatograms are provided separately in Supporting File 1.

A triglycine (G_3) motif was installed at the C-terminus of specified peptides through a bismaleimide scaffold (Figure 2A, Supporting Figure S5). Two back-to-back cysteine-maleimide reactions were used to install the C-terminal G_3 motif. First, ethylene-bis-maleimide (Santa Cruz Biotechnology #sc-358228) was reacted with G_3Cys with HPLC purification of the product. G_3-maleimide was then used to label the C-terminal cysteine on azide containing PTH1−11 peptides. Products were purified by using HPLC and characterized by mass spectrometry.

Sortase-Mediated Conjugation of Nbs with Peptides

Purified Nbs were used to prepare Nb-peptide conjugate using sortase-catalyzed conjugation, as previously described.[53] Details are provided in Supporting Methods. Mass spectra are provided separately in Supporting File 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISA was used to validate the binding of the dimeric peptide to specified Nbs, as described in Supporting Methods.

Fluorescence Resonance Energy Transfer (FRET)

An equimolar mixture (1 μM) donor and acceptor labeled samples (Nb_{ALFA}-FAM and Nb_{6E}-TMR) were incubated with varying concentrations of the template peptide (DP_{gly} or DP_{ahx}) in a 96 well flat-bottomed black plate. Each well was irradiated with the donor excitation wavelength of 485 nm. Signals were recorded using a Synergy Neo2 (BioTeK) plate reader in both donor and acceptor FRET channels, i.e., in the overlapping wavelength regions of the emission peak for both the donor and acceptor, respectively. The filter to measure the donor channel was set at an excitation wavelength of 485 nm, and the emission was measured at 528 nm. Similarly, the acceptor channel was set at an excitation wavelength of 530 nm, and the emission was measured at 590 nm. The fluorescence ratio was measured as the ratio of fluorescence intensities in donor and acceptor channels and plotted against the concentration of the dimeric peptide.

Assessment of Click Chemistry Using SDS-PAGE

Click reactions were performed at room temperature with a final concentration of 10 μM of each Nb bearing a click chemistry handle ($Nb_{6E}PEG_4DBCO$ and $Nb_{ALFA}biotinN_3$) in the presence or absence of equimolar template peptide (DP_{gly} or DP_{ahx}) in TBS. Reaction mixtures were quenched by adding a small peptide (G_3Lys(azide), 500 μM) to consume any residual DBCO containing probe. After quenching, samples were mixed with 4x Laemmli Sample Buffer (Biorad #1610747) supplemented with 100 mM dithiothreitol (DTT) and denatured by heating at 95 °C for 5 m. Samples were resolved on 15% polyacrylamide gels (generated in house) for 15 min at 90 V and then at 120 V for 1 h. Gels were stained with a PAGE-Blue protein staining solution overnight, destained, and imaged (Biorad ChemiDoc). Densitometric analysis of the high-molecular weight bands of the cross-linked product was done using Fiji software. Normalization and fold calculation were done relative to the mean integrated density of the high-molecular weight band from the control reaction quenched at T = 0.

Cell Culture

HEK293 cells stably expressing PTHR1 and a cAMP reporter gene were cultured in DMEM containing 10% fetal bovine serum (ThermoFisher no. A5669701) and 1X Pen-Strep (ThermoFisher #15140122) antibiotic. Cells were also checked and confirmed to be free of mycoplasma contamination.

Measurement of PTHR1-Mediated Signaling via $G_{\alpha s}$/cAMP in Cell-Based Assays

This assay was carried out using a previously reported method,[20] as described in Supporting Methods.

Data Analysis

Data were analyzed and prepared using MS-Excel and GraphPad Prism. GraphPad Prism was used to calculate and plot BRET ratios (515/410 nm) and AUC (area under the curve) values from kinetic studies. AUC was calculated in GraphPad Prism by integrating area under the curve from measurements performed throughout the 30 min read time. Data points and error bars correspond to the mean and SD of technical replicates, respectively.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00711.

Supporting Figures S1−S16; supporting methods; expression and purification of nanobodies; synthesis, derivatization, and characterization of peptides; sortase-mediated conjugation of Nbs with peptides; ELISA, GloSensor assay, BRET assay, cell viability assay (PDF)

MS characterization (peptides and Nb conjugates), HPLC chromatograms (peptides) (PDF)

AUTHOR INFORMATION

Corresponding Author

Ross W. Cheloha − *Laboratory of Bioorganic Chemistry, National Institutes of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, United States;* orcid.org/0000-0001-9871-8333; Email: ross.cheloha@nih.gov

Author

Shubhra Jyoti Saha − *Laboratory of Bioorganic Chemistry, National Institutes of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/jacsau.4c00711

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank M. Bouvier (University of Montreal) for the provision of plasmids used for BRET-based assays of β-arrestin. We acknowledge the NIDDK mass spectrometry core (J. Lloyd) for assistance. This work was supported by the NIH Intramural Research Program, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (NIDDK, 1ZIADK075157, R.W.C.) and funding from the NIH Director's Innovation Award. We acknowledge the NIH

Office of AIDS Research for Fellowship Support (S.J.S.). Figures were created in part using templates from BioRender.com.

REFERENCES

(1) Fralish, Z.; Chen, A.; Khan, S.; Zhou, P.; Reker, D. The Landscape of Small-Molecule Prodrugs. *Nat. Rev. Drug Discovery* **2024**, *23* (5), 365−380.
(2) Antonow, D. Fragment-Based Approaches and the Prospect of Fragmented Prodrugs. *Drug Discovery Today* **2010**, *15* (19), 801−803.
(3) Stanton, B. Z.; Chory, E. J.; Crabtree, G. R. Chemically Induced Proximity in Biology and Medicine. *Science* **2018**, *359* (6380), No. eaao5902.
(4) Gerry, C. J.; Schreiber, S. L. Unifying Principles of Bifunctional, Proximity-Inducing Small Molecules. *Nat. Chem. Biol.* **2020**, *16* (4), 369−378.
(5) Liang, F.-S.; Ho, W. Q.; Crabtree, G. R. Engineering the ABA Plant Stress Pathway for Regulation of Induced Proximity. *Sci. Signal.* **2011**, *4* (164), rs2−rs2.
(6) Miyamoto, T.; DeRose, R.; Suarez, A.; Ueno, T.; Chen, M.; Sun, T.; Wolfgang, M. J.; Mukherjee, C.; Meyers, D. J.; Inoue, T. Rapid and Orthogonal Logic Gating with a Gibberellin-Induced Dimerization System. *Nat. Chem. Biol.* **2012**, *8* (5), 465−470.
(7) Kennedy, M. J.; Hughes, R. M.; Peteya, L. A.; Schwartz, J. W.; Ehlers, M. D.; Tucker, C. L. Rapid Blue-Light−Mediated Induction of Protein Interactions in Living Cells. *Nat. Methods* **2010**, *7* (12), 973−975.
(8) Cheloha, R. W.; Gellman, S. H.; Vilardaga, J.-P.; Gardella, T. J. PTH Receptor-1 Signalling—Mechanistic Insights and Therapeutic Prospects. *Nat. Rev. Endocrinol.* **2015**, *11* (12), 712−724.
(9) Wacker, D.; Stevens, R. C.; Roth, B. L. How Ligands Illuminate GPCR Molecular Pharmacology. *Cell* **2017**, *170* (3), 414−427.
(10) Sexton, P. M.; Christopoulos, A. To Bind or Not to Bind: Unravelling GPCR Polypharmacology. *Cell* **2018**, *172* (4), 636−638.
(11) Wootten, D.; Christopoulos, A.; Marti-Solano, M.; Babu, M. M.; Sexton, P. M. Mechanisms of Signalling and Biased Agonism in G Protein-Coupled Receptors. *Nat. Rev. Mol. Cell Biol.* **2018**, *19* (10), 638−653.
(12) Devigny, C.; Perez-Balderas, A.; Hoogeland, B.; Cuboni, S.; Wachtel, R.; Mauch, C. P.; Webb, K. J.; Deussing, J. M.; Hausch, F. Biomimetic Screening of Class-B G Protein-Coupled Receptors. *J. Am. Chem. Soc.* **2011**, *133* (23), 8927−8933.
(13) Cheloha, R. W.; Fischer, F. A.; Woodham, A. W.; Daley, E.; Suminski, N.; Gardella, T. J.; Ploegh, H. L. Improved GPCR Ligands from Nanobody Tethering. *Nat. Commun.* **2020**, *11*, 2087.
(14) Sachdev, S.; Creemer, B. A.; Gardella, T. J.; Cheloha, R. W. Highly Biased Agonism for GPCR Ligands via Nanobody Tethering. *Nat. Commun.* **2024**, *15* (1), 4687.
(15) Cabalteja, C. C.; Sachdev, S.; Cheloha, R. W. Characterization of a Nanobody-Epitope Tag Interaction and Its Application for Receptor Engineering. *ACS Chem. Biol.* **2022**, *17* (8), 2296−2303.
(16) Cheloha, R. W.; Harmand, T. J.; Wijne, C.; Schwartz, T. U.; Ploegh, H. L. Exploring Cellular Biochemistry with Nanobodies. *J. Biol. Chem.* **2020**, *295* (45), 15307−15327.
(17) Noh, M.; Che, X.; Jin, X.; Lee, D.-K.; Kim, H.-J.; Park, D. R.; Lee, S. Y.; Lee, H.; Gardella, T.; Choi, J.-Y.; Lee, S. Dimeric R25CPTH(1−34) Activates the Parathyroid Hormone-1 Receptor in Vitro and Stimulates Bone Formation in Osteoporotic Female Mice. *eLife* **2024**, *13*, RP97579.
(18) Ling, J.; Cheloha, R. W.; McCaul, N.; Sun, Z.-Y. J.; Wagner, G.; Ploegh, H. L. A Nanobody That Recognizes a 14-Residue Peptide Epitope in the E2 Ubiquitin-Conjugating Enzyme UBC6e Modulates Its Activity. *Mol. Immunol.* **2019**, *114*, 513−523.
(19) Götzke, H.; Kilisch, M.; Martínez-Carranza, M.; Sograte-Idrissi, S.; Rajavel, A.; Schlichthaerle, T.; Engels, N.; Jungmann, R.; Stenmark, P.; Opazo, F.; Frey, S. The ALFA-Tag Is a Highly Versatile Tool for Nanobody-Based Bioscience Applications. *Nat. Commun.* **2019**, *10* (1), 4403.
(20) Braga Emidio, N.; Cheloha, R. W. Sortase-Mediated Labeling: Expanding Frontiers in Site-Specific Protein Functionalization Opens New Research Avenues. *Curr. Opin. Chem. Biol.* **2024**, *80*, No. 102443.
(21) Algar, W. R.; Hildebrandt, N.; Vogel, S. S.; Medintz, I. L. FRET as a Biomolecular Research Tool — Understanding Its Potential While Avoiding Pitfalls. *Nat. Methods* **2019**, *16* (9), 815−829.
(22) Kostic, M.; Jones, L. H. Critical Assessment of Targeted Protein Degradation as a Research Tool and Pharmacological Modality. *Trends Pharmacol. Sci.* **2020**, *41* (5), 305−317.
(23) Parvatkar, P.; Kato, N.; Uesugi, M.; Sato, S.; Ohkanda, J. Intracellular Generation of a Diterpene-Peptide Conjugate That Inhibits 14−3-3-Mediated Interactions. *J. Am. Chem. Soc.* **2015**, *137* (50), 15624−15627.
(24) Jaegle, M.; Wong, E. L.; Tauber, C.; Nawrotzky, E.; Arkona, C.; Rademann, J. Protein-Templated Fragment Ligations—From Molecular Recognition to Drug Discovery. *Angew. Chem., Int. Ed.* **2017**, *56* (26), 7358−7378.
(25) Brauckhoff, N.; Hahne, G.; Yeh, J. T.-H.; Grossmann, T. N. Protein-Templated Peptide Ligation. *Angew. Chem., Int. Ed.* **2014**, *53* (17), 4337−4340.
(26) Glassford, I.; Teijaro, C. N.; Daher, S. S.; Weil, A.; Small, M. C.; Redhu, S. K.; Colussi, D. J.; Jacobson, M. A.; Childers, W. E.; Buttaro, B.; Nicholson, A. W.; MacKerell, A. D., Jr.; Cooperman, B. S.; Andrade, R. B. Ribosome-Templated Azide−Alkyne Cycloadditions: Synthesis of Potent Macrolide Antibiotics by In Situ Click Chemistry. *J. Am. Chem. Soc.* **2016**, *138* (9), 3136−3144.
(27) Suzuki, T.; Ota, Y.; Kasuya, Y.; Mutsuga, M.; Kawamura, Y.; Tsumoto, H.; Nakagawa, H.; Finn, M. G.; Miyata, N. An Unexpected Example of Protein-Templated Click Chemistry. *Angew. Chem., Int. Ed.* **2010**, *49* (38), 6817−6820.
(28) Chaudhuri, R.; Prasanth, T.; Dash, J. Expanding the Toolbox of Target Directed Bio-Orthogonal Synthesis: In Situ Direct Macrocyclization by DNA Templates. *Angew. Chem., Int. Ed.* **2023**, *62* (7), No. e202215245.
(29) Jewett, J. C.; Bertozzi, C. R. Cu-Free Click Cycloaddition Reactions in Chemical Biology. *Chem. Soc. Rev.* **2010**, *39* (4), 1272−1279.
(30) Erickson, P. W.; Fulcher, J. M.; Spaltenstein, P.; Kay, M. S. Traceless Click-Assisted Native Chemical Ligation Enabled by Protecting Dibenzocyclooctyne from Acid-Mediated Rearrangement with Copper(I). *Bioconjugate Chem.* **2021**, *32* (10), 2233−2244.
(31) Rosenblatt, M.; Segre, G. V.; Tyler, G. A.; Shepard, G. L.; Nussbaum, S. R.; Potts, J. T. Identification of a Receptor-Binding Region in Parathyroid Hormone. *Endocrinology* **1980**, *107* (2), 545−550.
(32) Binkowski, B. F.; Butler, B. L.; Stecha, P. F.; Eggers, C. T.; Otto, P.; Zimmerman, K.; Vidugiris, G.; Wood, M. G.; Encell, L. P.; Fan, F.; Wood, K. V. A Luminescent Biosensor with Increased Dynamic Range for Intracellular cAMP. *ACS Chem. Biol.* **2011**, *6* (11), 1193−1197.
(33) Raimondi, S.; Porcari, R.; Mangione, P. P.; Verona, G.; Marcoux, J.; Giorgetti, S.; Taylor, G. W.; Ellmerich, S.; Ballico, M.; Zanini, S.; Pardon, E.; Al-Shawi, R.; Simons, J. P.; Corazza, A.; Fogolari, F.; Leri, M.; Stefani, M.; Bucciantini, M.; Gillmore, J. D.; Hawkins, P. N.; Valli, M.; Stoppini, M.; Robinson, C. V.; Steyaert, J.; Esposito, G.; Bellotti, V. A Specific Nanobody Prevents Amyloidogenesis of D76N B2-Microglobulin in Vitro and Modifies Its Tissue Distribution in Vivo. *Sci. Rep.* **2017**, *7*, 46711.
(34) Cheloha, R. W.; Fischer, F. A.; Gardella, T. J.; Ploegh, H. L. Activation of a G Protein-Coupled Receptor through Indirect Antibody-Mediated Tethering of Ligands. *RSC Chem. Biol.* **2021**, *2* (6), 1692−1700.
(35) Pleiner, T.; Bates, M.; Görlich, D. A Toolbox of Anti−Mouse and Anti−Rabbit IgG Secondary Nanobodies. *J. Cell Biol.* **2018**, *217* (3), 1143−1154.
(36) Avet, C.; Mancini, A.; Breton, B.; Le Gouill, C.; Hauser, A. S.; Normand, C.; Kobayashi, H.; Gross, F.; Hogue, M.; Lukasheva, V.; St-Onge, S.; Carrier, M.; Héroux, M.; Morissette, S.; Fauman, E. B.;

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789

Fortin, J.-P.; Schann, S.; Leroy, X.; Gloriam, D. E.; Bouvier, M. Effector Membrane Translocation Biosensors Reveal G Protein and Barrestin Coupling Profiles of 100 Therapeutically Relevant GPCRs. *eLife* **2022**, *11*, No. e74101.
(37) Sachdev, S.; Creemer, B. A.; Gardella, T. J.; Cheloha, R. W. Highly Biased Agonism for GPCR Ligands via Nanobody Tethering. *Nat. Commun.* **2024**, *15* (1), 4687.
(38) Braga Emidio, N.; Small, B. M.; Keller, A. R.; Cheloha, R. W.; Wingler, L. M. Nanobody-Mediated Dualsteric Engagement of the Angiotensin Receptor Broadens Biased Ligand Pharmacology. *Mol. Pharmacol.* **2024**, *105* (3), 260−271.
(39) Braga Emidio, N.; Cheloha, R. W. Semi-Synthetic Nanobody-Ligand Conjugates Exhibit Tunable Signaling Properties and Enhanced Transcriptional Outputs at Neurokinin Receptor-1. *Protein Sci.* **2024**, *33* (2), No. e4866.
(40) Brauckhoff, N.; Fang, L.; Haim, A.; Grossmann, T. N. Non-Enzymatic Protein Templates Amide Bond Formation and Provides Catalytic Turnover. *Chem. Commun.* **2023**, *59* (35), 5241−5244.
(41) Mocharla, V. P.; Colasson, B.; Lee, L. V.; Röper, S.; Sharpless, K. B.; Wong, C.-H.; Kolb, H. C. In Situ Click Chemistry: Enzyme-Generated Inhibitors of Carbonic Anhydrase II. *Angew. Chem., Int. Ed.* **2005**, *44* (1), 116−120.
(42) Whiting, M.; Muldoon, J.; Lin, Y.-C.; Silverman, S. M.; Lindstrom, W.; Olson, A. J.; Kolb, H. C.; Finn, M. G.; Sharpless, K. B.; Elder, J. H.; Fokin, V. V. Inhibitors of HIV-1 Protease by Using In Situ Click Chemistry. *Angew. Chem., Int. Ed.* **2006**, *45* (9), 1435−1439.
(43) Kang, S.; Davidsen, K.; Gomez-Castillo, L.; Jiang, H.; Fu, X.; Li, Z.; Liang, Y.; Jahn, M.; Moussa, M.; DiMaio, F.; Gu, L. COMBINES-CID: An Efficient Method for De Novo Engineering of Highly Specific Chemically Induced Protein Dimerization Systems. *J. Am. Chem. Soc.* **2019**, *141* (28), 10948−10952.
(44) Murray, C. W.; Rees, D. C. The Rise of Fragment-Based Drug Discovery. *Nat. Chem.* **2009**, *1* (3), 187−192.
(45) Wassarman, D. R.; Bankapalli, K.; Pallanck, L. J.; Shokat, K. M. Tissue-Restricted Inhibition of mTOR Using Chemical Genetics. *Proc. Natl. Acad. Sci. U. S. A.* **2022**, *119* (38), No. e2204083119.
(46) Martinko, A. J.; Tran, H. L.; Simonds, E. F.; Cooke, A. L.; Huang, Z.; Raguveer, S.; Agullet, J. P.; Prasad, S.; Going, C.; Marshall, L.; Park, T.; Banerjee, S.; Macdonald, R.; Jian, M.; Ng, K.; Palakodaty, A.; Kaur, M.; Ponce, A.; Tabrizi, M.; Hill, Z. B. A Bioorthogonal Antibody-Based Chemically-Induced-Dimerization Switch for Therapeutic Application. *bioRxiv*, April 11, 2023. DOI: 10.1101/2023.04.11.536272.
(47) Zhang, Z.; Fan, Q.; Luo, X.; Lou, K.; Weiss, W. A.; Shokat, K. M. Brain-Restricted mTOR Inhibition with Binary Pharmacology. *Nature* **2022**, *609* (7928), 822−828.
(48) Cupp, M. E.; Nayak, S. K.; Adem, A. S.; Thomsen, W. J. Parathyroid Hormone (PTH) and PTH-Related Peptide Domains Contributing to Activation of Different PTH Receptor-Mediated Signaling Pathways. *J. Pharmacol. Exp. Ther.* **2013**, *345* (3), 404−418.
(49) Spangler, S.; Bruchas, M. R. Tuning Biased GPCR Signaling for Physiological Gain. *Cell* **2017**, *171* (5), 989−991.
(50) Kolb, P.; Kenakin, T.; Alexander, S. P. H.; Bermudez, M.; Bohn, L. M.; Breinholt, C. S.; Bouvier, M.; Hill, S. J.; Kostenis, E.; Martemyanov, K. A.; Neubig, R. R.; Onaran, H. O.; Rajagopal, S.; Roth, B. L.; Selent, J.; Shukla, A. K.; Sommer, M. E.; Gloriam, D. E. Community Guidelines for GPCR Ligand Bias: IUPHAR Review 32. *Br. J. Pharmacol.* **2022**, *179* (14), 3651−3674.
(51) Dockerill, M.; Ford, D. J.; Angerani, S.; Alwis, I.; Dowman, L. J.; Ripoll-Rozada, J.; Smythe, R. E.; Liu, J. S. T.; Pereira, P. J. B.; Jackson, S. P.; Payne, R. J.; Winssinger, N. Development of Supramolecular Anticoagulants with On-Demand Reversibility. *Nat. Biotechnol.* **2024**, DOI: 10.1038/s41587-024-02209-z.
(52) Cabalteja, C. C.; Sachdev, S.; Cheloha, R. W. Rapid Covalent Labeling of Membrane Proteins on Living Cells Using a Nanobody−Epitope Tag Pair. *Bioconjugate Chem.* **2022**, *33* (10), 1867−1875.
(53) Cabalteja, C. C.; Sachdev, S.; Cheloha, R. W. Characterization of a Nanobody-Epitope Tag Interaction and Its Application for Receptor Engineering. *ACS Chem. Biol.* **2022**, *17* (8), 2296−2303.

https://doi.org/10.1021/jacsau.4c00711
JACS Au 2024, 4, 4780−4789