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Direct selection of DNA-encoded libraries for biased agonists of GPCRs on live cells

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Article

Direct Selection of DNA-Encoded Libraries for Biased Agonists of GPCRs on Live Cells

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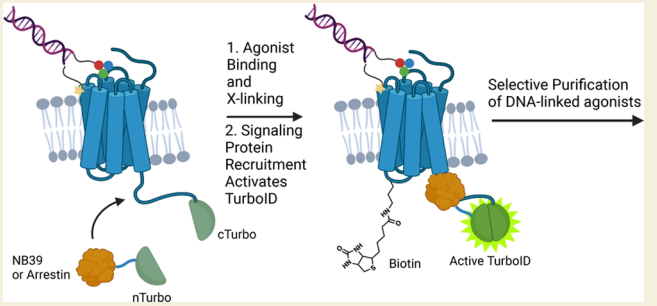
ABSTRACT: G protein-coupled receptors (GPCRs) are the largest superfamily of human membrane target proteins for approved drugs. GPCR ligands can have a complex array of pharmacological activities. Among these activities, biased agonists have potential to serve as both chemical probes to understand specific aspects of receptor signaling and therapeutic leads with more specific, desired activity. Challenges exist, however, in the development of new biased activators due, in part, to the low throughput of traditional screening approaches. DNA-encoded chemical libraries (DELs) dramatically improve the throughput of drug discovery by allowing a collective selection, rather than discrete screening, of large compound libraries. The use of DELs has been largely limited to affinity-based selections against purified protein targets, which identify binders only. Herein, we report a split protein complementation approach that allows direct identification of DNA-linked molecules that induce the dimerization of two proteins. We used this selection with a DEL against opioid receptor GPCRs on living cells for the identification of small molecules that possess the specific function of activation of either β-arrestin or G protein signaling pathways. This approach was applied to δ-, μ-, and κ-opioid receptors and enabled the discovery of compound [66,66], a selective, G-protein-biased agonist of the κ-opioid receptor (EC_{50} = 100 nM, E_{max} = 82%, G_i bias factor = 6.6). This approach should be generally applicable for the direct selection of chemical inducers of dimerization from DELs and expand the utility of DELs to enrich molecules with a specific and desired biochemical function.



KEYWORDS: *DNA-encoded libraries, G-protein-coupled receptors, biased agonists, chemical inducers of dimerization, split protein complementation*

INTRODUCTION

G-protein-coupled receptors (GPCRs), the largest target class of all FDA-approved drugs,[1] are essential regulators of numerous physiological processes.[2,3] Upon the binding of agonists, these receptors undergo conformational changes that lead to activation of heterotrimeric G-proteins and consequent downstream signaling.[4] In addition to G-protein signaling, agonist binding can cause desensitization, in which β-arrestin proteins are activated to interact with GPCRs, which blocks subsequent G protein activation and initiates additional, unique signaling pathways.[5−7] Signaling bias refers to the ability of GPCR agonists to preferentially activate either the G protein or β-arrestin signaling pathways.[8,9] In many cases, activation of one signaling pathway is desirable for therapeutic intervention, while the other one may be responsible for side effects.[3,10]

Opioid receptors (ORs) are among the class A subgroup of GPCRs.[11] ORs include the δ-, μ-, and κ-ORs (DOR, MOR, and KOR, respectively) and the nonclassical nociception OR (NOP).[12] For the MOR, some studies have indicated that the analgesic effects of agonists occur largely via G-protein signaling, while unwanted side effects occur through arrestin signaling.[13,14] A 2005 report of morphine treatment of β-arrestin 2 knockout mice showed both enhanced analgesia and dramatically reduced respiratory suppression and constipation.[14] This launched enthusiasm for development of G-protein-biased agonists of the MOR as safer analgesics. Results with such biased agonists have been mixed, however, and many subsequent studies have directly contradicted the hypothesis that arrestin signaling is solely responsible for negative side effects.[15] While the current situation of biased agonists of OR targets is muddled, biased agonism has become a key component of drug development for many GPCR targets.[16]

While several biased ligands of ORs (e.g., PZM21,[17] TRV130[18]) have been developed, the discovery of biased agonists remains challenging using traditional screening approaches. Alternatively, researchers have employed computa-

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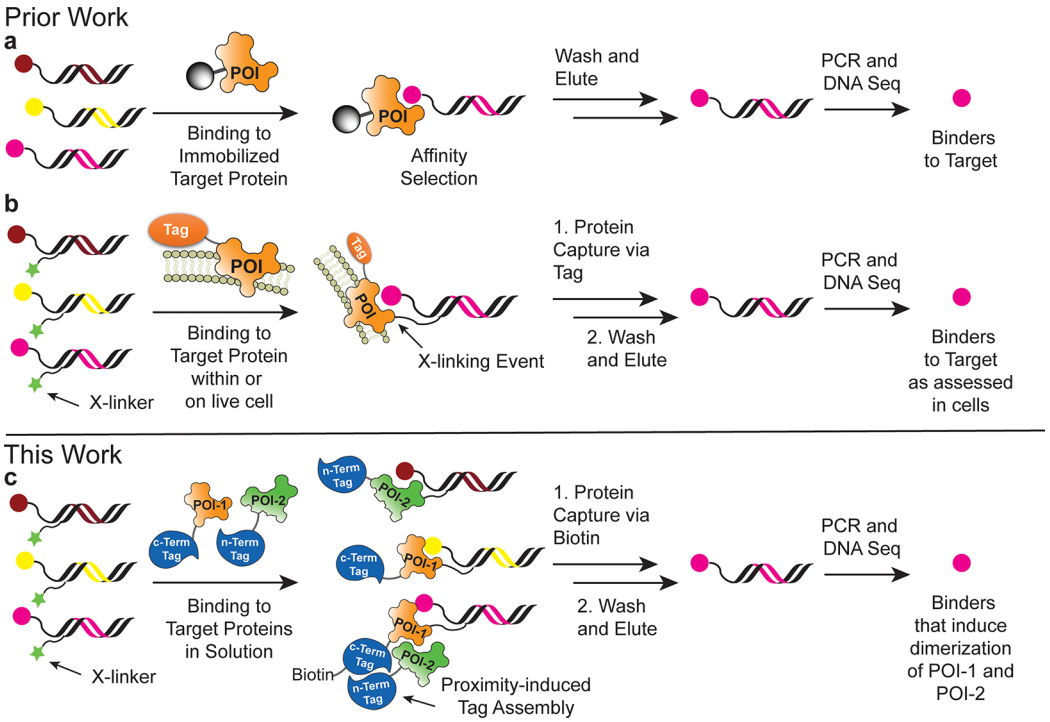
Scheme 1. DEL Selection Assays[a]



[a](a) Traditional DEL selection assays involve an affinity selection against a solid-phase immobilized protein target to identify protein binders. (b) Prior work has utilized affinity cross-linking to assess the binding of DEL ligands to protein targets within or on live cells. After affinity labeling, ligands are purified by a fusion protein tag on the protein target. (c) Split protein tag reconstitution allows conditional labeling of a protein target with a purification handle (biotin) upon the binding of a ligand that induces protein dimerization. When combined with covalent cross-linking, this allows selective purification of DEL members that are both protein binders and inducers of protein dimerization.

tional and structure-based discovery approaches to develop biased agonists at GPCRs.[3,17] Recently, a structure-based evolution approach explored the signaling profiles of PZM21 derivatives, which led to the discovery of FH210, an MOR agonist with improved selectivity and more pronounced G protein bias.[19] Before the implementation of such campaigns, however, extensive structural information of the target is required. Besides structure-based strategies, Lerner and co-workers developed an autocrine-based signaling system that, when paired with fluorescence-based cell sorting, identified biased agonists of GPCRs from genetically expressed peptide libraries. With this system, a G-protein-biased agonist of the glucagon-like peptide-1 receptor, PL5, was identified.[20]

DNA-encoded library (DEL) technology is changing the landscape of drug discovery research, but its utility in developing lead compounds for membrane proteins remains underexplored. Similar to prior work with cell-based selection of aptamers and phage-displayed peptides against cell surface target proteins,[21] there are a few examples of DEL selections which involve direct binding to live cells followed by repeated centrifugation and washing. In 2011, the Bradley group selected a peptide nucleic acid (DNA mimetic)-encoded peptide library against cell surface receptors.[22] Israel et al. performed cell-based DEL selections against the NK3 tachykinin GPCR.[23] The Neri group selected bivalent DELs against carbonic anhydrase IX-expressing tumor cells.[24] Zhao and co-workers successfully used natural product-based DELs to perform selection against an immobilized angiotensin II type I receptor.[25] Recently, the Li group used affinity labeling to label membrane proteins with an ssDNA tag to guide DEL members to a specific target and increase their binding affinity through avidity, which enabled selections against endogenous membrane proteins.[26] Besides cell-based selections, the Lefkowitz group has performed affinity selection of a DEL against purified, detergent-solubilized β2-adrenergic receptors (β2ARs).[27] In these examples, however, the DEL selection assays employed distinguish only binders from non-binders to the membrane protein targets. A method that directly uses a selection to identify ligands with distinct biochemical function beyond binding is challenging yet highly desirable for drug discovery. Given the chemical diversity achievable with DELs, such an approach could contribute greatly to the structure–activity relationship understanding of GPCR signaling bias.

We previously reported a covalent cross-linking-based approach that enabled DEL selections to be performed against protein targets within and on living cells (Scheme 1b).[28] After the binding event occurs between a protein target and DNA-linked ligands, affinity labeling covalently traps protein–ligand interactions in, or on, live cells prior to the purification of the protein target via a genetically encoded tag (e.g., HaloTag, SnapTag). We hypothesized that using covalent cross-linking together with split protein tag complementation would enable a signaling pathway specific selection assay for enrichment of biased GPCR agonists from DELs. The use of genetically encoded, split protein tags has been used previously in GPCR assays to identify agonists that recruit specific signaling proteins. This was initially demonstrated with split green fluorescent protein (GFP) pairs for fluorescent assays for indication of biased agonists.[29] More recent assays have employed additional split protein tags including β-galactosidase[30] and nanoluciferase (NanoLuc).[31] In this work, we have developed a selection assay that combines covalent cross-

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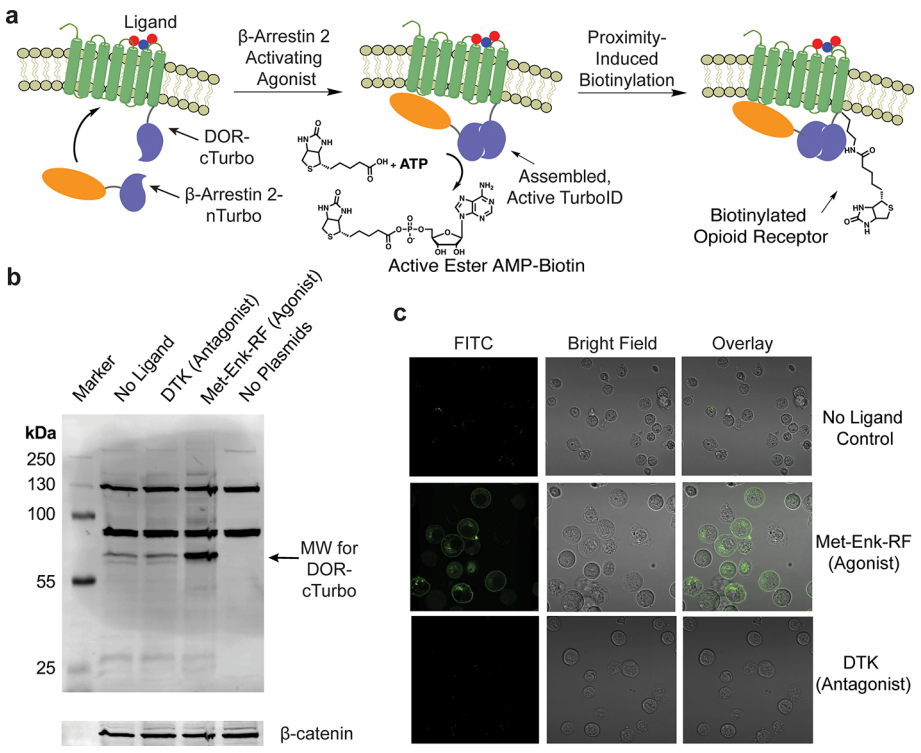

Figure 1. A split-TurboID protein complementation approach specifically biotinylates the DOR when treated with a *β*-arrestin 2 agonist. (a) Scheme of the split protein complementation approach. Ligands that are *β*-arrestin agonists will recruit the *β*-arrestin protein tagged with the N-terminal half of TurboID. The proximity allows the protein halves to assemble and become active leading to biotinylation of nearby proteins. (b) Streptavidin blot of SDS-PAGE of Expi293 cell lysates verified that biotinylation of the DOR by TurboID is dependent on treatment with a *β*-arrestin agonist (Met-Enk-RF). Protein biotinylation was detected by LI-COR IRDye 680RD-streptavidin. The two bands present in all lanes correspond to endogenously biotinylated proteins: 3-methylcrotonyl CoA carboxylase (75 kDa) and pyruvate carboxylase (130 kDa). (c) Confocal fluorescence imaging of cells expressing DOR-cTurbo and *β*-arrestin 2-nTurbo incubated with *β*-arrestin 2 antagonist DTK or agonist Met-Enk-RF. Neutravidin-FITC was used to detect biotinylated proteins.

linking with conditional labeling of the target protein with a purification tag (biotin) upon split protein complementation (Scheme 1c). We demonstrate the approach as a general selection for chemical inducers of dimerization. Using DNA-linked, model ligands of known bias, we validated these selection assays for both G-protein- and *β*-arrestin-biased agonism of opioid GPCRs on live cells. Lastly, we constructed a ca. 10,000-membered combinatorial DEL and applied our approach to the DOR, MOR, and KOR in parallel selections for multiple functions on the target proteins when expressed on live cells.

RESULTS

To develop a general approach for direct selection of DNA-encoded ligands that induce protein dimerization, we initially used the dimerization of FK506 binding protein (FKBP) and FKBP-rapamycin binding domain (FRB)[32] as a model system together with a split SnapTag.[33] SnapTag is a commonly used fusion protein based on O-6-methylguaninine-DNA methyltransferase that enables covalent tagging of an active site thiol with *O*-benzylguanine (BG) derivatives.[34] Based on a published split SnapTag protein complementation assay,[33] we validated rapamycin-dependent reconstitution of an active SnapTag through dimerization of FKBP-cSnap and FRB-nSnap with subsequent labeling with benzylguanine-*fluorescein amidite* (FAM) (BG-FAM) (Figure S1a,c). A non-dimerizing ligand [synthetic ligand for FKBP (SLF)][35] did not induce protein labeling. In a selection assay against 6-His-FKBP-cSnap alone (Scheme S1), both DNA-linked rapamycin and SLF were similarly enriched (6600-fold and 4000-fold, respectively) over a non-ligand control (Figure S1d). Test selections were conducted to selectively enrich DNA-linked ligands that induce protein dimerization, where reconstituted SnapTag is labeled with BG-biotin for a subsequent streptavidin bead purification (Scheme S2). DNA-linked rapamycin was enriched (20−140-fold), while DNA-linked SLF was not (∼2-fold) (Figure S1d). Further evaluation of this approach in live HEK293F cells was unsuccessful, however. Both confocal microscopy (Figure S2a,c) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell lysates (Figure S2b) indicated self-assembly of the SnapTag in the absence of the rapamycin dimerizer. Consistent with these observations, test selections with a DNA-linked DOR agonist and DOR-cSnap and *β*-arrestin 2-nSnap constructs achieved only 2-fold enrichment (Figure S3). These results agree with a report published in the course of this work which observed self-association (in the absence of induced proximity) of these same SnapTag split fragments when expressed in mammalian cells.[36]

We then evaluated a split TurboID tag to enable the installation of a purification tag on the target that is conditional upon the binding of a protein dimerizer. TurboID is an engineered biotin ligase that catalyzes the biotinylation of proteins in proximity (∼10 nm) via the release of an active ester adenosine monophosphate-biotin.[37] Split-TurboID (cut site: L73/G74) consists of two inactive fragments of TurboID.[38,39] Based on a recent report on the use of split-

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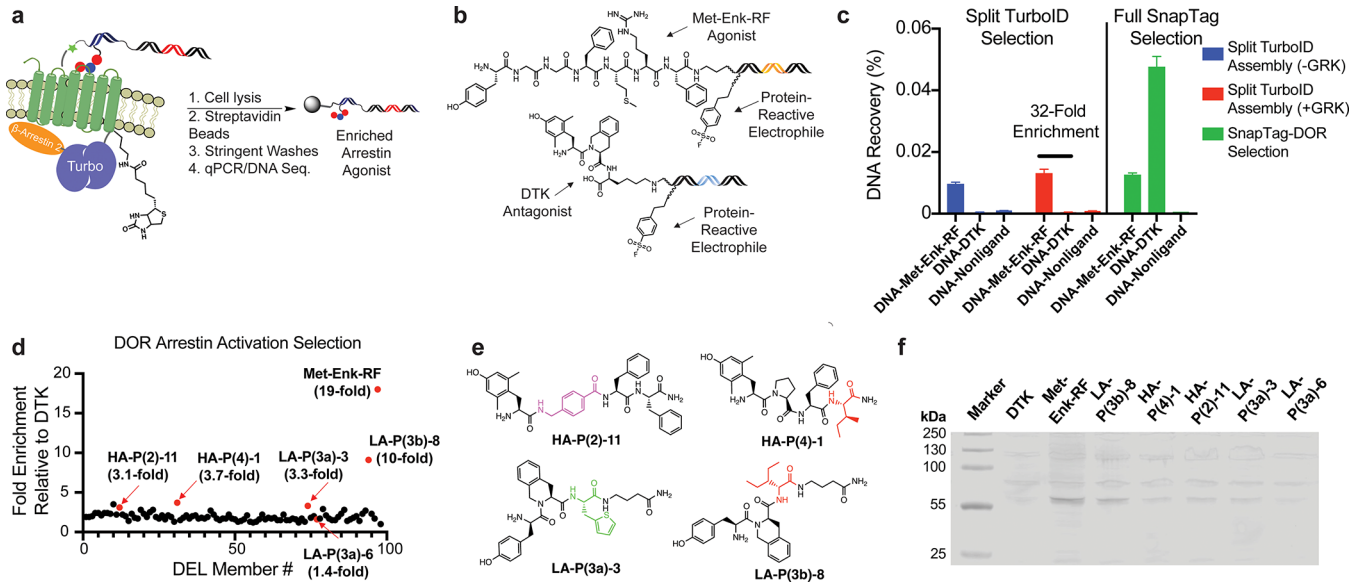

Figure 2. Selective enrichment of DNA-linked DOR β-arrestin 2 agonists. (a) Split-TurboID-based protein complementation approach to select for DNA-linked β-arrestin 2 agonists on live cells. An active TurboID leads to receptor biotinylation, which enables selective purification of cross-linked DNA-linked ligands that arrest agonists. (b) Tested DNA constructs in mock selection assays. (c) Recovery and enrichment of DNA constructs in test selections. Recovery values are reported with the mean from two replicates with standard deviation (SD). (d) Illumina DNA sequencing enrichment results normalized to the DTK antagonist with compounds selected for follow-up indicated. (e) Structures of selected compounds synthesized off-DNA. (f) In-gel streptavidin staining of an SDS-PAGE gel of cell lysates showing biotinylation of the DOR induced by selected compounds.

TurboID with a GPCR and β-arrestin,[38] we moved forward directly to test this approach for identifying ligands that activate the β-arrestin pathway of the DOR in cell-based assays. We constructed DOR-cTurboID and β-arrestin 2-nTurboID and co-expressed these proteins in HEK293F cells. β-Arrestin 2 agonists lead to proximity-induced reassembly of the TurboID, which then leads to the biotinylation of the DOR (Figure 1a). We verified the β-arrestin agonist-dependent biotinylation of the DOR in a gel-based assay (Figure 1b). Met-Enk-RF (a β-arrestin-biased DOR agonist)[40] treatment markedly increased the biotinylation level of a band at the expected molecular weight of the DOR in comparison to the absence of the ligand or treatment with the tripeptide Dmt-Tic-Lys[41] (DTK, a high-affinity DOR antagonist). No band was observed in untransfected cells. We additionally tested a split HaloTag as an alternative system;[42] however, labeling tests with similar split HaloTag constructs were unsuccessful (Figure S4). Confocal microscopy of split TurboID constructs showed that fluorescence was clearly detectable on the outer membrane of live cells only in Met-Enk-RF-treated cells (Figure 1c) upon neutravidin-FAM staining but not in DTK-treated samples. Taken together, these results demonstrated that the split-TurboID tag had good "switchability" and low levels of self-assembly (Figure 1b, no ligand and DTK lanes). Thus, we proceeded with the split TurboID-based approach to develop our selection assays.

We performed a selection assay for enrichment of DNA-linked β-arrestin 2 agonists of the DOR, as outlined in Figure 2a. DOR-cTurboID and β-arrestin 2-nTurboID are expressed in HEK293F cells. G protein-coupled receptor kinase 2 (GRK2) was co-expressed with the two proteins (plasmid ratio: 1:4:4),[43] as phosphorylation of GPCRs by GRKs leads to high-affinity β-arrestin binding.[44] DNA-linked ligands bind to an OR and are covalently cross-linked to DOR-cTurboID. Ligands that are β-arrestin agonists will recruit the β-arrestin protein tagged with the N-terminal half of TurboID, leading to a reconstitution of an active TurboID, which then leads to proximity-induced biotinylation. The biotinylated DOR can be captured on streptavidin beads; after stringent washes, enriched ligands are identified by DNA sequencing. Because HEK293F cells themselves provide sufficient adenosine triphosphate and biotin to initiate the biotinylation reaction, this approach only requires a simple incubation of a DEL with cells to directly identify ligands with specific biochemical functions. In our test selection, we observed 32-fold enrichment for the agonist (DNA-linked Met-Enk-RF, Figure 2b,c), while no enrichment was observed for the antagonist (DNA-linked DTK, Figure 2b,c). In contrast, due to its high affinity to the DOR, DNA-linked DTK was highly enriched in an affinity-based selection against the full SnapTag-DOR protein target (Figures 2c and S5). This difference indicates that the split TurboID-based protein complementation approach allows the direct selection for ligands not only on the basis of binding to the target but also on the basis of ability to bind as an agonist that activates a specific downstream signaling pathway.

To further evaluate the assay performance, we sought to identify β-arrestin 2 agonists from a previously published collection of 96 DNA-encoded compounds containing single monomer substitutions of a high-affinity parental peptide Dmt-Pro-Phe-Phe ($K_i \approx 28$ nM)[45] and a low-affinity parental peptide Tyr-Tic ($K_i \approx 240$ nM).[46] We doped the DNA-DTK antagonist as a negative control and DNA-linked Met-Enk-RF as a positive control. DNA sequencing of the selection results showed ≈20-fold enrichment of Met-Enk-RF relative to the DTK control (Figure 2d). A single library member [LA-P(3b)-8] exhibited high enrichment (≈10-fold). We chose five library members for off-DNA synthesis and testing (Figure 2e). These included the highest enricher and three additional molecules that showed slight enrichment above the background (3−4-fold) and also a molecule that showed effectively no

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enrichment as a negative control. We used gel-based assays to test these molecules for arrestin activation of the DOR in cells with the same split-Turbo constructs used in the selection (Figure 2f). We observed biotinylation levels that generally correlated with the DNA sequencing results. As expected, these enrichment results contrast dramatically with the previously published selection for binding only,[28] which shows a wide variety of enrichment levels over a ∼10-fold range. The result that few compounds showed enrichment in the arrestin recruitment assay is likely due to design of the library, which is based on DOR antagonists.

To develop an analogous assay for G-protein activation, we used nanobody 39 (Nb39), a camelid nanobody that is specific for the G-protein activated state of ORs.[47] State-specific nanobodies have been widely used to study GPCR conformational dynamics,[48−50] and Nb39 has been used in a similar split protein complementation assay with NanoLuc to monitor G-protein activation of the MOR.[51] Based on the crystal structure of the Nb39-stabilized active state of the KOR[52] (Figure 3a,

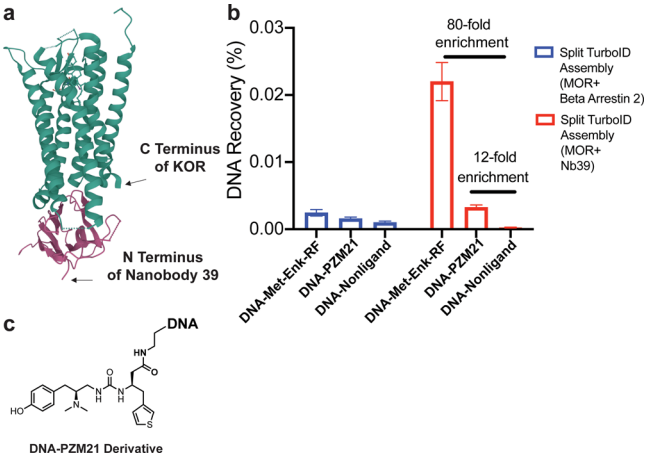

Figure 3. Selection assay for G-protein agonists of the MOR. (a) Crystal structure of the Nb39-stabilized active state of the KOR (PDB: 6B73). (b) A qPCR-based selection assay using DNA-linked ligands shows enrichment of the G-protein-biased agonists (Met-Enk-RF and PZM21) using the Nanobody 39-split TurboID assay and little to no enrichment in similar arrestin activation selection assays. Recovery values are reported with the mean from two replicates with standard deviation (SD). (c) Structure of the DNA-linked PZM21 derivative.

PDB 6B73), we fused the N-terminal portion of TurboID to the N-terminus of Nb39 and the C-terminal portion of TurboID to the C-terminus of ORs, such that the split TurboID halves will be brought into close proximity to reconstitute a functional TurboID. To validate our G-protein activation assay, we performed test selections against the MOR using a DNA-linked Met-Enk-RF peptide and a DNA-linked PZM21 derivative (Figure 3c), a G-protein-biased MOR agonist.[17] Unlike the arrestin bias with the DOR, the Met-Enk-RF peptide shows strong G-protein bias for the MOR.[40] In the quantitative polymerase chain reaction (qPCR) selection assay, we observed 80-fold enrichment of the DNA-Met-Enk-RF and 12-fold enrichment of the DNA-PZM21 over the non-ligand control (Figure 3b), which is consistent with the expected difference in potency. The results for MOR selections together with the results with the DOR show that application of these assays with DELs can be used to discover OR ligands and determine their molecular mechanism of action on the receptor as either β-arrestin-recruiting agonists or G-protein-activating agonists.

With the aim of discovering novel biased ligands, we constructed a combinatorial DEL containing 9216 (96^2) small molecules using split-and-pool synthesis (Figure 4). As OR ligands such as PZM21 and fentanyl are both structurally simple and have high potency,[53] our library was constructed in just two encoding steps. The library was focused on to expand on the PZM21 structure by inclusion of urea linkages and also the fentanyl class of OR ligands which contains a piperidine core (Figure 4). In the first step, we incorporated one or two monomers of an Fmoc amino acid or a peptoid, which was then capped as an amide or urea in the second step. To enable the formation of urea linkage, we used di-2-pyridyl carbonate activation of a DNA amine,[54] followed by the addition of excess amine. The successful formation of urea linkage on-DNA was confirmed by liquid chromatography−mass spectrometry (Figure S6). To synthesize fentanyl-type pharmacophores on-DNA, we performed reductive amination of two piperidine cores with various aldehydes and ketones (Figure S7).

After the synthesis of the targeted DEL, we assayed the library for both β-arrestin and G-protein signaling agonism against three targets (DOR, MOR, and KOR) using the split TurboID-based protein complementation approaches. Met-Enk-RF and DTK were doped into the library to serve as controls, and qPCR results indicated enrichment values consistent with previous test selections (Figure S8). Figure 5 shows 2D scatter plots of DNA sequencing results of the top 100 enriched compounds in each of the selections. The maximum fold enrichment values observed were modest, ranging from a ∼3.6-fold MOR with Nb39 to ∼20-fold with the KOR and Nb39, yet these values are generally consistent with the assays with control compounds. Patterns of similarly enriched compounds are observed as lines in these plots. These patterns are distinct between the various selections. Several compounds containing building block #62, which corresponds to the dimethyltyrosine urea derivative contained within PZM21, were enriched in selections against both the DOR and MOR.

To verify the selection results, six compounds (Figure S9) were selected for off-DNA resynthesis. We selected three compounds with enrichment profiles that indicated bias for β-arrestin signaling and three compounds that indicated G-protein bias by Nb39 recruitment. To validate these as hits, bioluminescence resonance energy transfer (BRET)-based assays[49] were conducted to measure β-arrestin recruitment and G-protein activation (Figures S10 and 11, respectively). We observed noteworthy agonism for one of the six compounds, [66, 66] (Figure 6a). Consistent with the DNA sequencing results, this compound shows selective G-protein-biased agonism of the KOR (100 nM EC_{50}) with 10-fold and 20-fold selectivity over the DOR and MOR, respectively (Figure 6c). We additionally validated the receptor selectivity of [66,66] for Nb39 recruitment in a streptavidin blot assay (Figure 6b). Compound [66,66] exhibits substantial signaling bias as it shows much weaker activation of β-arrestin at the KOR (Figures 6c and S10). This compound has high structural similarity to known KOR agonists (Figure 6d).[55] The urea linkage, however, is unique. Compared to similar compounds, [66, 66] exhibits a dramatically more "efficacy-dominant" mode of signaling bias than the "affinity-dominant" profile of

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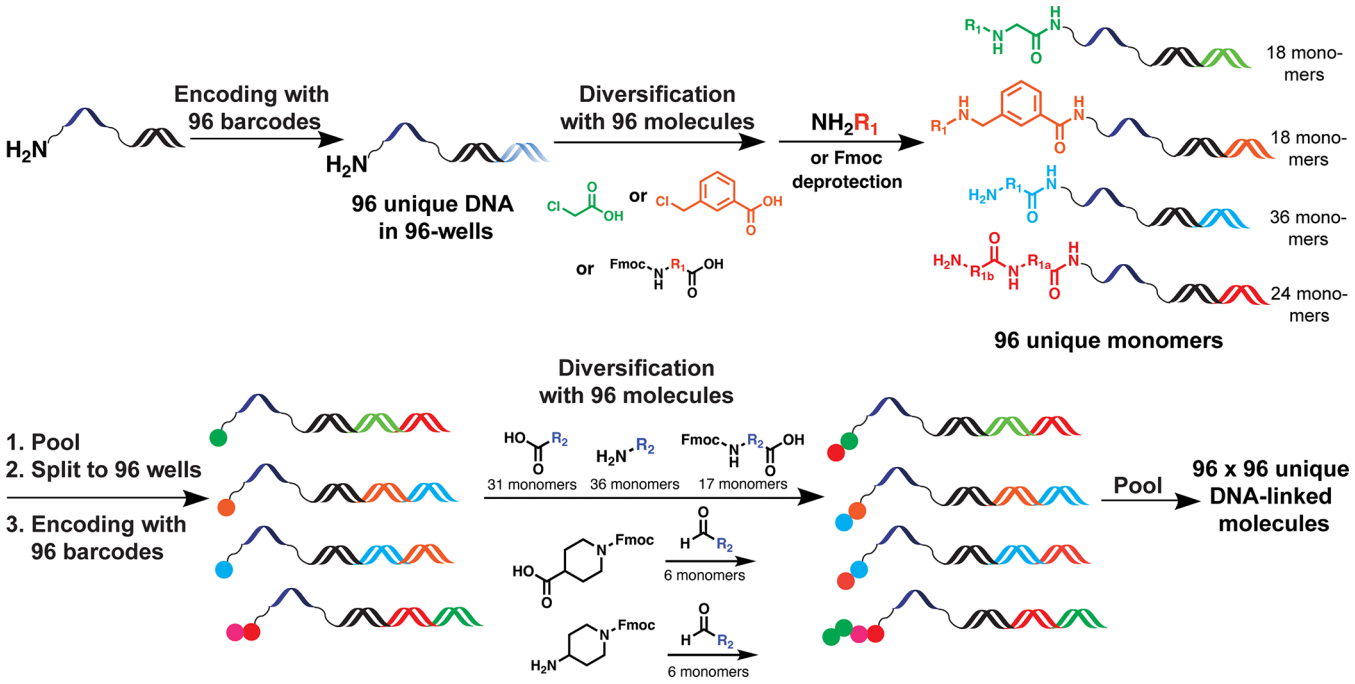

Figure 4. Split-and-pool, combinatorial synthesis of an OR-targeted DEL.

homologous compounds.[56] For the additional two compounds ([68,77] and [87,44]) selected for Nb39 recruitment, we did observe significant, yet modest, G-protein agonism, which did not show appreciable selectivity (Figure S11).

The three compounds selected for arrestin agonism did not show activity in the BRET assays. Gel-based, streptavidin blot assays were used to additionally investigate arrestin activation of the DOR and MOR in cells expressing the same split-Turbo constructs used in the selection. These compounds did show biotinylation of these ORs in gel-based assays (Figure S12); however, the activity and selectivity were modest. The co-expression of GRK2 in our selection assay, which was not present in the BRET assay, may increase sensitivity. To test this, we included GRK2 in arrestin 2 BRET assays. However, we did not observe any notable changes in the activity of tested compounds (Figure S13). This suggests that GRK2 is not the major factor that contributes to the discrepancy between BRET and selection assays. The signal of the selection assay relies upon compound-induced biotinylation of ORs, which can accumulate over time via enzymatic turnover. In similar gel-based assays that also harness the power of enzymatic turnover, β-arrestin activating compounds such as LA-P(3b)-8 and [80,49] consistently showed strong activation of β-arrestin at the DOR (Figure S14).

Given the modest success of our previously selected six compounds, we further performed binding selections using the enzyme-mediated proximity biotinylation approach against the three ORs (Figures S15 and S16) using a full UltraID tag fused to the N-terminus of the receptors on the cell exterior.[57] For these binding assays, we observed more consistent profiles among the three receptors and similar maximal enrichments of ~6-fold. Integration of this data with the previous selections should facilitate elimination of false positives and indicate molecules that both bind with high affinity and are agonists of the receptor.

As G-protein-biased agonists have been sought after as novel treatments for pain, we sought to further identify ligands that selectively activate G-protein signaling. We chose five compounds that were enriched in both the binding selections and Nb39-specific selections and not enriched in arrestin recruitment selections. We selected one compound ([68,66]) that was enriched in selections against both the DOR and MOR. Given the higher fold enrichments and prior success with [66, 66], the remaining four compounds were chosen from the KOR selections (Figure 7a). In testing off-DNA compounds in BRET assays, we observed significant G-protein agonism for all compounds (see Figures S17–S19 for the full collection of plots). The activity of compound [3, 84] was very weak, however. Compound [68,66] did show the most potent G-protein activation among the five compounds for the DOR and weak activation of the MOR (Figure S17) but did not show selectivity over the KOR. Among the selected KOR hit compounds, [71,66] was particularly efficacious and potent (EC_{50} = 33 nM, E_{max} = 100%) for G-protein activation at the KOR with over 50-fold selectivity toward the DOR and MOR. In BRET-based β-arrestin assays, [71,66] weakly activated β-arrestin signaling of the KOR with an EC_{50} of 1.4 μM (Figures 7b and S19).

To quantify ligand bias, we repeated BRET assays of the top KOR G-protein-biased agonists [66, 66] and [71. 66] along with U50,488 as a reference compound (Figure S20). Bias factors were determined using the Blank and Leff operational model, as previously described.[58] [66, 66] shows G-protein bias with a bias factor of 6.6, while [71, 66] is a more balanced agonist with a factor of 1.3. Both compounds are mixtures of stereoisomers with [66, 66] being two enantiomers and [71, 66] being four stereoisomers. Synthesis and testing of each isomer may yield more potent and biased compounds. Of note, recent studies have shown that assessing ligand bias by comparing G-protein activation versus arrestin binding to the receptor tends to overestimate G-protein signaling.[59] While the Blank and Leff operational model used in this work has been used to address this issue, this correction may not be sufficient to measure bias for weak partial agonists.

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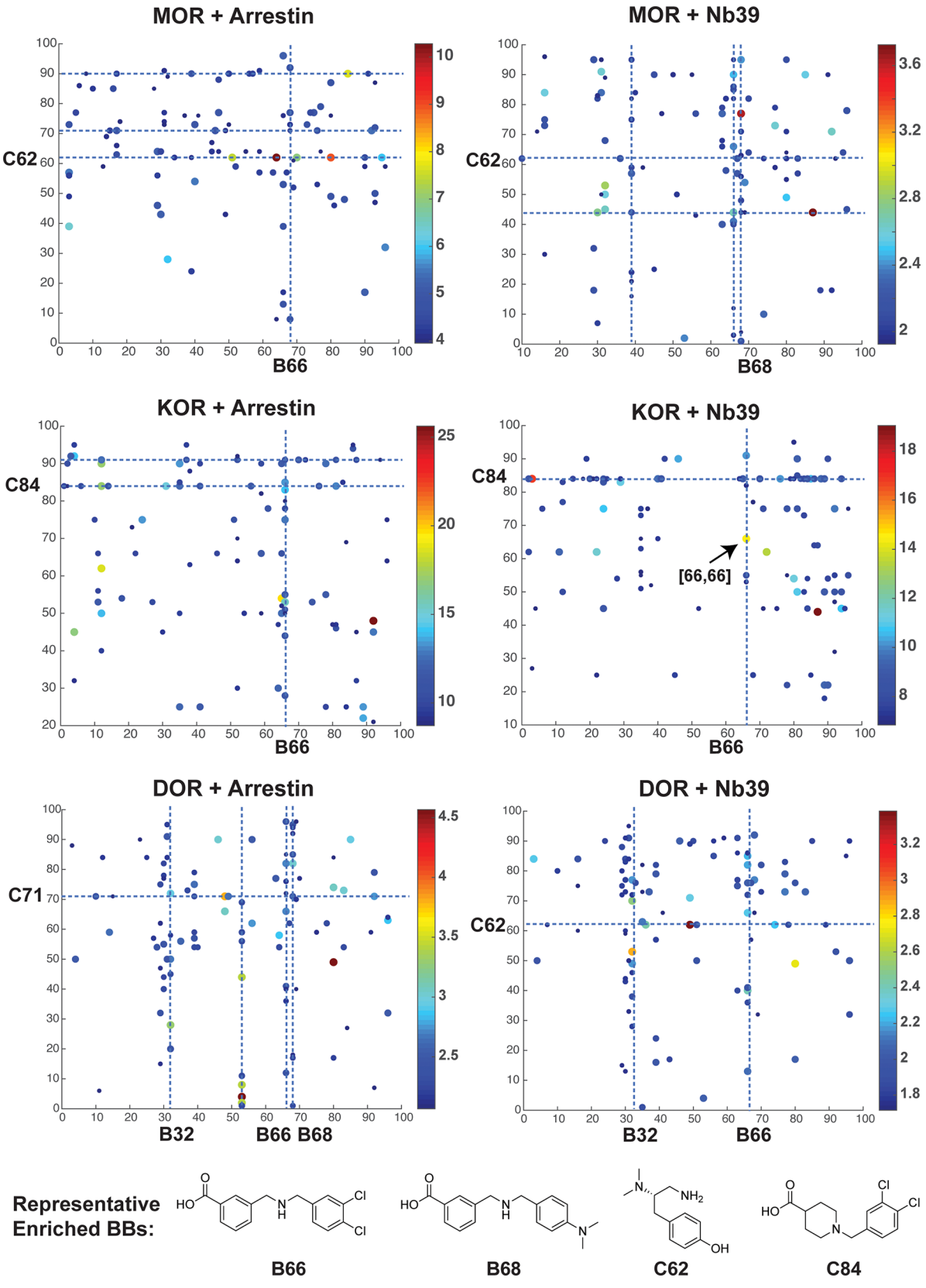

Figure 5. 2D scatter plots of DEL sequencing results. Top 100 compounds enriched in selections are shown. The color scale represents the enrichment factors for each library member which were calculated as (post-selection abundance)/(pre-selection abundance). The *x* and *y* axes represent the building block used in the first and second steps in the synthesis, respectively. See Table S3 for building blocks. Dot size is scaled relative to enrichment and is color-coded by enrichment.

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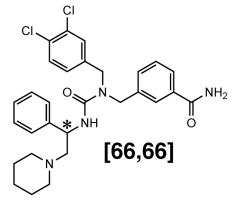
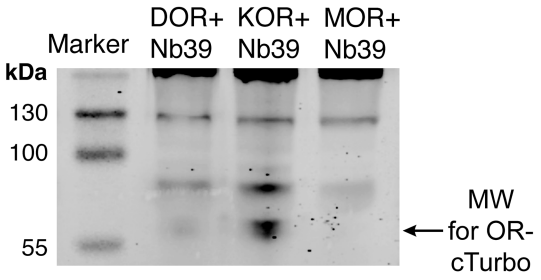


	DOR	MOR	KOR
Enrich. in Arrestin Selection	2.7	1.7	2.2
Enrich. in Nb39 Selection	2.3	2.4	15

b



c

Receptor	Gi		β Arrestin 2	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
DOR	ND	-	ND	-
MOR	ND	-	NA	-
KOR	100 ± 20	82 ± 2	1400 ± 600	23 ± 2

d

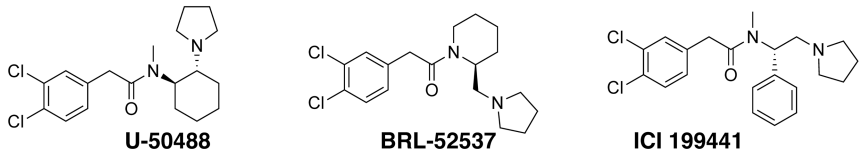


Figure 6. Off-DNA hit validation of an Nb39-activating compound for the KOR. (a) Structure and enrichment values of compound [66, 66]. (b) In-gel streptavidin staining of SDS-PAGE gel of cell lysates showing biotinylation of receptors induced by compound treatment. (c) EC_{50} and E_{max} values of [66, 66] obtained from BRET-based β-arrestin/Gi protein recruitment assays at the DOR, KOR, and MOR. Values are reported with the mean from three replicates ± standard error measurements (SEMs). (d) Structurally similar KOR agonists.[55] EC_{50} values were marked as not determined (ND) for compounds that showed weak activation but did not give full curves. Compounds that gave no significant activation are marked as no activation (NA).

CONCLUSIONS

In summary, we developed an approach that enables the use of DELs to identify small molecules with the specific biochemical function of inducing protein dimerization, in contrast to conventional selections that are based on binding only. This approach capitalizes on the power of affinity labeling and the split protein complementation. While recent innovations in DEL technology have expanded its utility to live cell protein targets, selection assays of DELs with live cell targets for functions beyond binding are yet to be demonstrated. Here, we have applied unique selection assays with DELs against three major therapeutic drug targets in a large-scale parallel analysis for multiple functions of 9612 molecules. The assays identified novel scaffolds and chemotypes, including compound [66,66], a selective and highly G-protein-biased agonist of the KOR, and compound [71,66] that potently and selectively activates G-protein signaling of the KOR. Compared with those of more traditional approaches, the cost, effort, and infrastructure required in DELs are reduced dramatically. Thus, the development of DEL assays for specific cellular functions, beyond simple protein binding, is highly desirable.[60−62] Further optimization of this approach will improve the signal to noise ratio of the assay (i.e., fold enrichment) and enable the application of more complex DELs and higher confidence in hit determination. This method will serve as a convenient tool for the rapid identification of biased lead compounds for medicinal chemistry optimization and chemical probes to explore the biology of GPCRs and other cell signaling targets.

METHODS

Cell Line and Culture Conditions

Expi293F suspension cells were purchased from Thermo Fisher Scientific (catalog A14527). Expi 293-F cells were cultured in a 25 mL Erlenmeyer flask with 4 mL of Expi293 Expression Medium. Flasks were shaken at 130 rpm using an orbital shaker (KJ-201 BD) placed in a 37 °C incubator with 8% CO_2 and >85% relative humidity.

Transient Transfection

The transient transfection was performed following the manufacturer's protocols with slight modifications. One day prior to transfection, cells were split to a final density of 2.5 × 10^6 viable cells/mL and were allowed to grow overnight. On the next day, cells reached a density of ≈4.5−5 × 10^6 viable cells/mL with >95% viability. 10 million cells were harvested, washed with phosphate buffered saline (PBS), and resuspended in 4 mL of fresh Expi293 Expression Medium (final density: 2.5 × 10^6 viable cells/mL). 4 μg of each plasmid DNA was diluted with 240 μL of Opti-MEM I reduced serum medium (Thermo Fisher Scientific, catalog 31985062). For selections that enrich β-arrestin 2 agonists, 1 μg of GRK2 was included. 18 μL of ExpiFectamine 293 Reagent (Thermo Fisher Scientific) was diluted with 240 μL of Opti-MEM I reduced serum medium and was allowed to incubate at room temperature for 5 min prior to the initiation of the plasmid DNA complex reaction. The diluted ExpiFectamine 293 Reagent was then added to diluted plasmid DNA and gently mixed by pipetting three times. The ExpiFectamine 293/plasmid DNA complexes were allowed to incubate at room temperature for 15 min and were transferred dropwise to cells. Cells were cultured in 25 mL Erlenmeyer flasks and shaken at 130 rpm using an orbital shaker (KJ-201 BD) in a 37 °C incubator with 8% CO_2 and >85% relative humidity. 22 h post-

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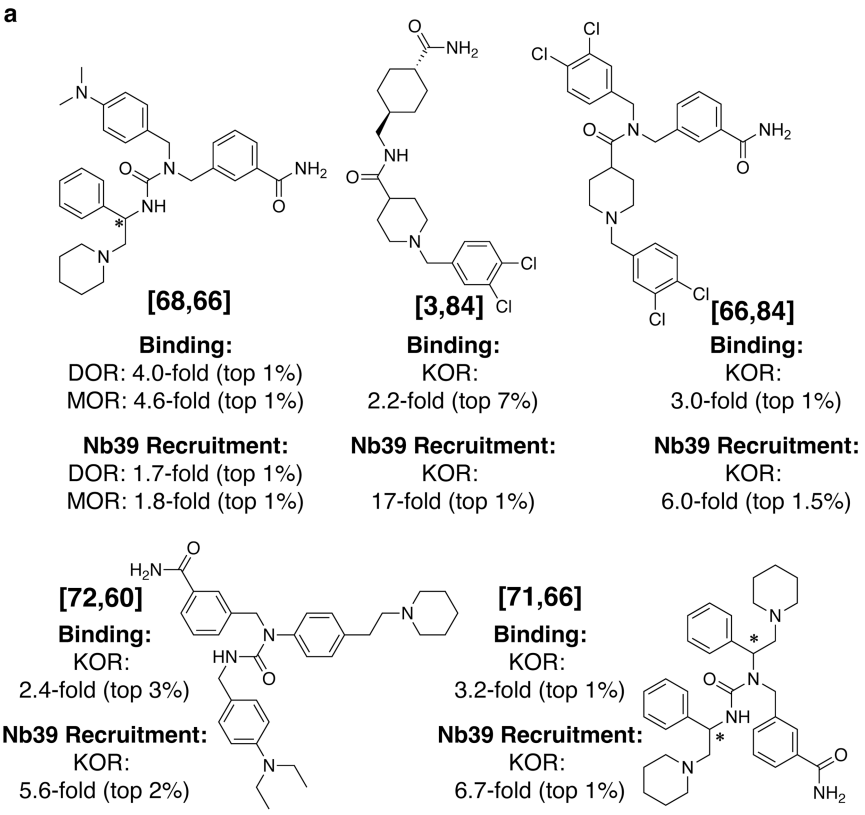
a



b

Compound	KOR-Gi		KOR-β Arrestin 2	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
U50488	6 ± 1	-	400 ± 40	-
[68,66]	1200 ± 300	20 ± 7	-	-
[71,66]	34 ± 1	100 ± 0.2	1400 ± 130	62 ± 2
[72,60]	700 ± 20	42 ± 8	-	-
[66,84]	N.D.	-	-	-

Figure 7. Off-DNA hit validation of compounds enriched from both binding selection and Nb39-specific selections. (a) Structures and enrichment values of selected hit compounds. (b–d) EC_{50} and E_{max} values of hit compounds obtained from BRET-based β-arrestin/Gi protein recruitment assay at the DOR, MOR, and KOR. Values are reported with the mean from three replicates ± SEMs. EC_{50} values were marked as not determined (ND) for compounds that showed weak activation but did not give full curves.

transfection, 24 μL of ExpiFectamine 293 Transfection Enhancer 1 and 240 μL of ExpiFectamine 293 Transfection Enhancer 2 were added to the flasks and allowed to be shaken in the incubator for another 24 h. Cells were then harvested for selection experiments.

Live Cell Selections to Enrich β-Arrestin 2 or Nb39 Activating Ligands on Opioid Receptors

Approximately 2.5 million Expi293F cells (Thermo Fisher Scientific, catalog A14527) expressing indicated constructs were harvested, washed twice with PBS, and resuspended in 50 μL of cell culture medium. The DEL (≈100 pmol total amount) was incubated with cells in a 37 °C incubator for 15 min, followed by the addition of 2 μL of 100 μM ssDNA-linked sulfonyl fluoride. The mixture was allowed to incubate on a rotator at 37 °C for 30 min. After incubation, cells were washed with 50 μL of PBS twice. To effectively extract and purify membrane proteins, cells were lysed with the Mem-PERTM Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Catalog 89842) or 200 μL of homemade radioimmunoprecipitation assay buffer [recipe: 1% Tergitol (NP-40), 0.1% deoxycholate, 0.5% SDS, 120 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1× Roche complete protease inhibitor, and 1% Sigma phosphatase inhibitor cocktail 3]. The supernatant of the cell lysis was recovered by centrifugation at 16,000×g at 4 °C for 25 min. To capture biotinylated ORs, the supernatant was diluted with 400 μL of PBS before the addition of 5 μL of pre-washed NanoLink streptavidin magnetic beads (Solulink, catalog M-1002-010). Bovine serum albumin (BSA) and salmon sperm DNA (Thermo Fisher Scientific) were added to a final concentration of 0.5 mg/mL to minimize background binding to beads. After incubation at room temperature for 1 h, the magnetic beads were separated and the supernatant removed. Beads were then washed with PBS containing 0.5 mg/mL salmon sperm DNA, 0.5 mg/mL BSA, and 0.1% SDS three times. For the fourth wash, beads were washed with PBS. Finally, beads were resuspended in 50 μL of water and boiled below 95 °C for 5 min. The elution samples were PCR-amplified to add specific sample-identifying sequencing adapters for DNA sequencing. qPCR analysis was performed with the pre-selection and post-selection samples to quantify the enrichment values of positive controls before sequencing (Figure S8).

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Quantitative PCR (qPCR) Analysis

qPCR reactions were performed in 384-well plates (VWR, catalog 82051-464) with a total volume of 12 μL in each well. Each 12 μL of reaction contains 3 μL of the diluted pre-selection/post-selection sample, 3 μL of 1 μM corresponding forward and reverse primers, and 6 μL of Applied Biosystems PowerUp SYBR Green Master Mix (Fisher Scientific, catalog A25742). All qPCR reactions were performed in duplicate with the thermal cycling procedure as described before.[28]

DNA Sequencing and Data Analysis

Illumina next-generation sequencing primers and indices were added to elution samples from each selection by PCR. The primers containing specific Illumina adaptor sequences and index sequences are listed in Table S2. PCR reactions were performed using the following condition: 1× Thermo Scientific Dream Taq buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM Illumina forward primer, 0.5 μM Illumina reverse primer, and 0.025 u/μL Dream Taq DNA polymerase for 18 PCR cycles (94 °C for 3 min, 94 °C for 15 s, annealing at 58 °C for 15 s, extension at 72 °C for 15 s, and a final extension of 72 °C for 5 min). The PCR reactions were pooled, purified by homemade solid-phase reversible immobilization (SPRI) magnetic beads, and quantified by UV absorbance at 260 nm. Sequencing reads were parsed from FASTQ files to text files containing only the 40-mer barcode sequences as a linear sequence for each read. Reads of the variable sequence region were matched to barcodes as individual 40 mers using MATLAB. Only sequences with ≥36 out of 40 correct base matches were assigned to barcodes. Reads were then collated and filtered to count total read numbers of each library member. Enrichment factors for each library member were calculated as (post-selection abundance)/(pre-selection abundance).

Off-DNA Hit Validation with Western Blot

10 μM hit compounds were incubated with 1 million cells expressing the same split-TurboID constructs used in selections. For validation experiments with arrestin-activation ligands, GRK2 was co-expressed with split-TurboID constructs. Following incubation, cells were washed twice with PBS and lysed using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, catalog 89842). Membrane proteins were separated on 10% SDS-PAGE gels and transferred onto a poly(vinylidene fluoride) membrane. Blots were then blocked in 3% BSA in *Tris buffered saline Tween-20* for 30 min and incubated with the anti-β-catenin antibody produced in the rabbit antibody (MilliporeSigma, catalog C2206) for 16 h at 4 °C. Blots were then washed with PBS with Tween 20 (PBST) three times and incubated with the anti-rabbit-IRDye800CW secondary antibody and streptavidin-IRDye680RD (LI-COR) for 1 h at room temperature. Blots were washed three times with PBST and imaged on an Odyssey Imaging System (LI-COR).

Off-DNA Hit Validation by Bioluminescence Resonance Energy Transfer (BRET) Assay

A 1:1:1:1 ratio of individual ORs (KOR, DOR, or MOR)/Gα-RLuc8/Gβ/Gγ-GFP2 DNA diluted in Opti-MEM were transfected into HEK-293T cells to determine the coupling between the ORs and G proteins. A 1:5 DNA ratio of individual ORs (KOR, DOR, or MOR) engineered to fuse with Renilla luciferase (RLuc8) at the C-terminus and β-arrestin 2 ligated to mVenus at the N-terminus were transfected into HEK-293T cells to measure the recruitment of β-arrestin 2. Transit 2020 was used as a transfection reagent and prepared in a ratio of 2 μl of Transit/1 μg of DNA and then incubated for 40 min before being added to the cells. 24 h post-transfection, Dulbecco's modified Eagle's medium that contained 1% dialyzed fetal bovine serum was used to plate the transfected cells at a density of 50,000 cells per 200 μl per well into 96-well white clear bottom cell culture plates (coated with poly-L-lysine). Next day, 60 μL per well of the RLuc substrate (5 μM coelenterazine 400a) or RLuc substrate (5 μM coelenterazine h) was added to the cells and incubated for 5 min in the dark at room temperature to activate Gα protein or arrestin, respectively, after aspirating their media and washing them with 60 μL per well of a drug buffer (1× HBSS and 20 mM HEPES, pH 7.4) per well. Following this, 30 μL per well of the (3×) compound that was prepared in buffer (1× HBSS and 20 mM HEPES, pH 7.4) supplemented with 0.3% BSA was added and incubated for another 5 min. For the measurement of the BRET ratio for Gα protein activation and β-arrestin 2 recruitment, a Mithras LB940 multimode microplate reader was immediately used. This was done by detecting the ratio of the GFP2 emission at 510 nm to Rluc emission at 395 nm for Gα protein activation and the ratio of mVenus emission at 485 nm to Rluc emission at 530 nm for β-arrestin 2 recruitment for 1 s per well. The potency and efficacy of the examined drugs were calculated using GraphPad Prism 9 software after plotting and normalization of the BRET ratios against various drug concentrations.

Quantification of Functional Selectivity (Bias Factor)

To quantify the degree of ligand's [66,66] and [71,66] bias numerically with U50,488 used as the reference compound, the dose−response data set for $G_{\alpha i1}$-protein-mediated signaling (BRET2) and β-arrestin 2-mediated signaling (BRET1) was plotted in GraphPad Prism 9.0 using the Black and Leff operational model.[9] Subsequently, the magnitude of transduction coefficients [log (τ/K_A)] in activating a particular signaling pathway for [66,66] and [71,66] was obtained, where τ and K_A represent the agonist's efficacy and the agonist equilibrium dissociation constant, respectively. For each ligand, the $G_{\alpha i1}$ transduction coefficient $\Delta\log\ (\tau/K_A)_{\text{ligand1/G}\alpha i1\text{pathway}}$ was subtracted from the β-arrestin 2 transduction coefficient $\Delta\log\ (\tau/K_A)_{\text{ligand1/}\beta\text{-arrestin2pathway}}$ to estimate the log bias, $\Delta\Delta\log\ (\tau/K_A)$. Accordingly, the bias factor was generated by calculating $10^{\Delta\Delta\log(\tau/KA)}$ as previously described.[58b]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.2c00674.

Synthetic procedures, cloning, cell culture, streptavidin blot, library construction, selection details, off-DNA hit synthesis, characterization, and validation data (PDF)

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Notes

The authors declare no competing financial interest.

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