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Modulating β Arrestin-2 Recruitment at the δ- and μ-Opioid Receptors Using Peptidomimetic Ligands

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[%]Electronic Supporting Information (ESI) is available: Experimental details for the synthesis of, characterization of, and determination of purity for compounds **1b–1g**, detailed pharmacological

procedures, additional pharmacological characterization, stability data, computational procedures, additional computational data and figures.

Keywords: Leu-enkephalin; delta opioid receptor; mu opioid receptor; beta-arrestin; biased signaling

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Abstract

μ Opioid receptors agonists provide potent and effective acute analgesia; however, their therapeutic window narrows considerably upon repeated administration, such as required for treating chronic pain. In contrast, bifunctional μ/δ opioid agonists, such as the endogenous enkephalins, have potential for treating both acute and chronic pain. However, enkephalins recruit β-arrestins, which correlate with certain adverse effects at μ- and δ-opioid receptors. Herein, we identify the C-terminus of Tyr-ψ[(*Z*)CF=CH]-Gly-Leu-enkephalin, a stable enkephalin derivative, as a key site to regulate bias of both δ- and μ-opioid receptors. Using *in vitro* assays, substitution of the Leu^5 carboxylate with amides (NHEt, NMe_2, $N^{Cy}Pr$) reduced β-arrestin recruitment efficacy through both the δ- and μ-opioid, while retaining affinity and cAMP potency. For this series, computational studies suggest key ligand-receptor interactions that might influence bias. These findings should enable discovery of a range of tool compounds with previously unexplored biased μ/δ opioid agonist pharmacological profiles.

Introduction

Though μOR (μ-opioid receptor) agonists are highly effective analgesics, particularly in acute and subacute peri-operative settings, they are not recommended for treating chronic pain due to concerns about analgesic tolerance, as well as an increase in likelihood and severity of adverse μOR-mediated side effects, including constipation, dependence and respiratory depression.[1] Additionally, activation of the μOR is less able to overcome the adaptive changes that occur in patients suffering from chronic pain. In sharp contrast to μOR agonism, δOR (δ-opioid receptor) agonism less effectively induces acute analgesia,[2] though δOR agonists display utility in chronic pain settings, including migraine, inflammatory and neuropathic pain.[2–5] However, there are currently no FDA-approved δOR agonists, in part because δOR agonists have the potential for inducing seizures.[6] If the μOR and δOR adverse effects could be reduced or avoided, a bifunctional μOR/δOR agonist could produce analgesia for acute and chronic pain.[7] Such an agonist could be useful in patients suffering from chronic pain, particularly for patients with cancer or arthritis who experience episodes of breakthrough pain[8–10].

The exact mechanisms for the described μOR and δOR-related adverse effects have been an issue of debate. First, recruitment of β-arrestin (β-Arr) 2 has been hypothesized as possible underlying cause for both δOR-induced seizures, as well as μOR-induced respiratory depression, constipation, and the development of analgesic tolerance.[11–13] β-Arr 2 KO mice have been reported to have diminished μOR side effects[14] or no impact,[15] while phosphorylation-deficient mutant μORs also still exhibit μOR side effects, with the exception of tolerance.[16] The therapeutic window for μOR agonists has been positively correlated with G-protein bias, [17,18] but a recent study argued that the correlation was driven by G-protein signaling efficacy rather than bias.[19] This study has spurned an alternate hypothesis that gives more significance to partial

agonism especially when receptor reserve is limited.[20,21] A third hypothesis emphasizes intracellular Golgi signaling as a contributor to μOR-induced adverse effects, which is minimized with peptide-based agents.[22] According to these concepts, the creation of peptidic opioids, that do not readily activate intracellular ORs and that exhibit reduced efficacy for both β-Arr 2 *and* G-protein signaling, may provide antinociception with improved therapeutic windows. Some μOR/δOR bifunctional agonists indeed display antinociception with reduced tolerance, dependence, locomotor activation and self-administration relative to classical morphinans.[7,23–25] Though thus far, development of these μOR/δOR dual agonists has largely ignored β-Arr 2 recruitment, which makes it impossible to predict the contribution of low β-Arr 2 recruitment to the reduced side effect profile in the context of dual agonism of μOR/δOR. In a single exception, UFP-505, a μOR/δOR dual agonist, activates β-Arr 2 through the μOR, but underrecruits β-Arr 2 at δOR, and also only exhibits partial agonist G-protein activity at δOR.[26] Thus, μOR/δOR dual agonists with a range of well-characterized β-Arr 2 profiles are essential to validate μOR/δOR dual agonism as a desired pharmacological profile.

Peptides have historically served as ligands for studying opioid pharmacology, and in many cases the rapid and modular synthesis of peptides has enabled the delivery of analogs with novel profiles. We recently showed that small modifications of Phe[4] can alter arrestin recruitment and μOR/δOR potency and selectivity of Leu[5]-enkephalin (Leu-Enk, YGGFL), an endogenous δOR opioid peptide,[27] while other δOR pentapeptides exist that display G-protein biased signaling profiles albeit with low potency.[28] As such, derivatization of peptides can facilitate the study of biased-signaling in relation to desired μOR and/or δOR-mediated antinociception and undesired adverse effects. Herein, we derivatize the carboxyl-terminal region of previously reported Leu[5]-Enk pepidomimetics[29,30] with the goal of delivering a set of opioid

peptides with varying degrees of β-Arr 2 recruitment, in particular with limited μOR β-Arr 2 recruitment, as such compounds remain unidentified. Further, computational modeling points to key ligand-target interactions that regulate β-Arr 2 recruitment at both receptors, which provides insight for designing next-generation analogs with precisely tuned pharmacological profiles for studying antinociceptive potency and adverse effect profiles signal-biased μ/δ opioids.

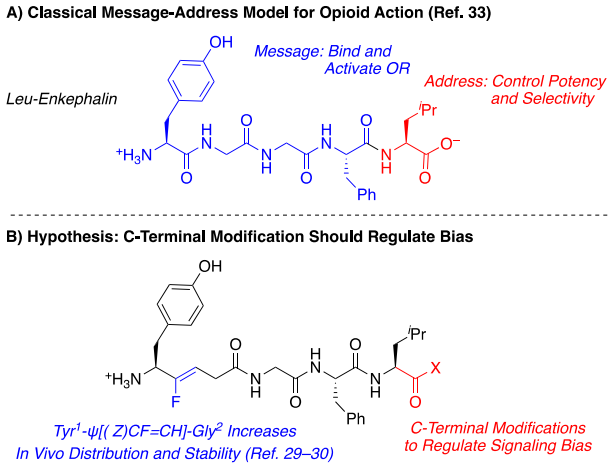


Figure 1. Designing Leu-Enk Analogs with Decreased β-Arr 2 Recruitment. (A) According to the classical "Message-Address" model for opioid action, the C-terminal residue regulates opioid selectivity. By extension of this model, modifications to this position might also regulate bias at the δOR (cAMP vs. β-Arr 2). (B) The present work exploits C-terminal modifications in the "Address" domain to deliver biased δOR agonists with low β-Arr recruitment at both the δOR and μOR.

Design Considerations: To deliver a series of peptide-based signal-biased δOR/μOR agonists, we initially explored Leu-Enk, which acts at the δOR with 1–5-fold higher binding affinity over μOR and >1000-fold over κOR,[31,32] and that has served as a starting point for decades worth of medicinal chemistry efforts to study OR pharmacology. In a seminal paper

from 1981, Chavkin and Goldstein introduced the "message-address" concept of opioid peptide binding to opioid receptors.[33] According to this model, Tyr[1]-Gly[2]-Gly[3]-Phe[4], the common backbone of Leu[5]-Enk, Met[5]-Enk and dynorphin constitute the "message" that to recognizes and binds to opioid receptors, and that amino acids at the fifth position and beyond contribute to the "address" portion of the peptide that confers potency and receptor selectivity (Figure 1A).[33] Though this hypothesis was developed prior to recognition of opioid-induced β-Arr signaling, we speculated that the message-address model might apply to the concept of biased ligands, specifically that C-terminal modifications of Leu[5]-Enk might reduce β-Arr recruitment potency at ORs (Figure 1B). In support of this hypothesis, replacement of Leu[5] with aza-β-homoleucine or cycloleucine residues biases signaling toward G-protein coupling at the δOR (2–5 fold bias factor), though these ligands still overrecruit β-Arr though the μOR,[34] which may lead to undesired adverse effects. Nonetheless, we envisioned that alternate modifications near the C-terminus might further regulate bias at both the μOR and δOR, specifically by weakening charged interactions between the anionic C-terminus of the ligand and cationic residues in receptor and by increasing steric bulk in this region (Figure 2). To explore this hypothesis, we initiated studies using Leu-enk derivatives bearing the Tyr-ψ[(*Z*)CF=CH]-Gly substitution that improves stability, physicochemical and distribution properties relative to the parent peptide, while still delivering a single digit nanomolar δOR agonist activity (Figure 2).[29,30]

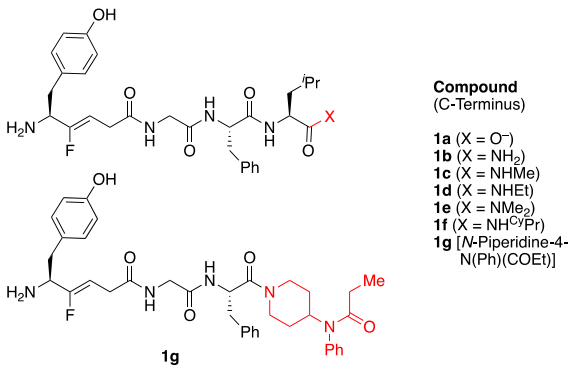
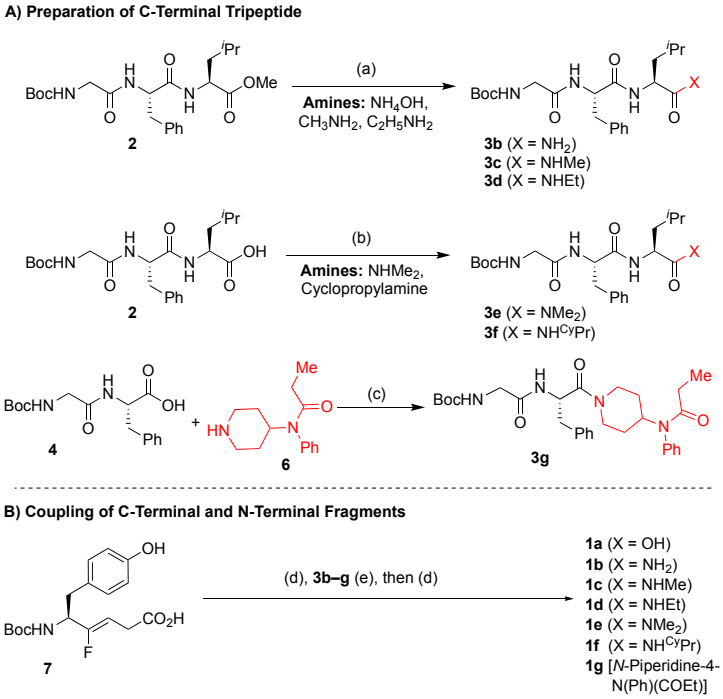


Figure 2. C-Terminal Substituted Analogs Synthesized and Pharmacologically Characterized.

Synthesis of Analogs: Analogs were generally prepared using microwave-assisted solution phase coupling chemistry using a Boc-protection strategy that has previously been demonstrated to deliver peptides in high purity (Scheme 1).[35] C-terminal functionalized tripeptides (**3b–d**) were accessed from the corresponding methyl esters (**2a**). To access compounds **3b–d**, reaction of the amine with the corresponding ester afforded the tripeptides in suitable yields, though these conditions did not afford bulkier intermediates **3e–f**. Thus to access **3e–f**, we reacted the amines with the corresponding acid (**2b**) using coupling with *N,N'*-diisopropylcarbodiimide (DIC) and *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide (HONB) under microwave (MW) irradiation. These conditions also effectively coupled Boc–Gly–Phe–OH (**4**) with *N*-Piperidine-4-N(Ph)(COEt) (**6**) to afford **3g**. These tripeptides were deprotected using HCl in 1,4-dioxane, then coupled on to Tyr-ψ[(*Z*)CF=CH]-Gly–OH[29,30] using DIC/HONB with *N,N*-diisopropylethylamine (DIEA) under MW irradiation and subsequently deprotected (Scheme 1B). Purification by reversed phase HPLC provided analytically pure samples for pharmacological evaluation.



Scheme 1. Synthesis of Analogs **1a**–**g**. Reagents and Conditions: (a) Amine : MeOH (1:1), rt, 14 h; (b) Amine, DIC, HONB, DMF, 60 °C, 30 min, MW; (c) DIC, HONB, DMF, 60 °C, 30 min, MW; (d) 4N-HCl in 1,4-Dioxane, 15 °C, 30 min; (e) DIC, HONB, DIEA, DMF, 60 °C, 30 min, MW.

Results and Discussion

C-terminal substitution of Tyr-ψ[(*Z*)CF=CH]-Gly-Leu-Enk with various alkyl amides (Figure 2) delivered a series of compounds with sub-μM binding affinities at both δOR and μOR (Table 1), G-protein coupling activities comparable to the parent carboxylate **1a** (Figure 3A,C), and interestingly demonstrating a range of β-Arr recruitment activities with clear structure-function trends (Figure 3B,D).

Table 1. Binding Affinities at δOR and μOR for C-Terminal Analogs of Tyr-ψ[(*Z*)CF=CH]-Gly-Leu-Enk

Compound	pK_i±SEM (δOR)	K_i (nM)	pK_i±SEM (μOR)	K_i (nM)	Binding Selectivity (δOR vs μOR)
1a (O^-)	7.59±0.2	25.6	7.37±0.1	42.7	1.7
1b (NH_2)	7.03±0.2	94.4	8.15±0.1	7.07	0.1
1c (NHMe)	7.25±0.1	55.9	8.00±0.2	9.92	0.2
1d (NHEt)	7.26±0.1	54.7	7.70±0.1	20.0	0.4
1e (NMe_2)	6.59±0.1	255.1	7.07±0.1	85.4	0.3
1f ($NH^{Cy}Pr$)	6.99±0.1	103.5	7.58±0.2	26.1	0.3
1g [Pip-N(Ph)(COEt)]	6.43±0.1	372.4	6.43±0.1	368.1	1.0
Leu^5-Enk	8.95±0.1	1.12	8.69±0.1	2.07	1.8

Using standard competition radioligand binding assays and [^{3}H]DPDPE or [^{3}H]DAMGO as radioligands, C-terminal substituted analogs **1b–f** engaged both the δOR and μOR within an order of magnitude of parent compound **1a**, with bulky analog **1g** binding with slightly lower affinities (Table 1). However, a clear trend emerged with analogs bearing at least one H-bond donor-acceptor pair (e.g. NH_2, NHMe, NHEt, $NH^{Cy}Pr$; **1b–d**, **f**) possessing better binding affinities relative to analogs bearing bulky NMe_2 and Pip-N(Ph)(COEt) (**1e**, **1g**) substituents. Further, analogs **1b–1f** bearing C-terminal amides preferentially bound to the μOR (selectivities: 0.1–0.4), which contrasts the parent analogs and Leu-Enk that preferentially bound to the δOR (selectivities: 1.7–1.8), or analog **1g** that bound to the two receptors with equal affinities (1.0–1.2).

Despite these binding trends, analogs **1b–g** activated both the δOR and μOR with within an order of magnitude of the potency as the parent using the GloSensor assay (Table 2). In general, the potency for the peptides to recruit β-Arr 2 at δOR was 10-fold lower than for the peptides to inhibit cAMP at δOR (Table 2), which matches previous findings.[27] Despite their similar binding profiles (Table 1) and potencies inhibiting cAMP (Figure 3A,C), the bulky C-terminal substituted enkephalin peptides weakly recruited β-Arr 2 at δOR and μOR (Table 2, Figure

3B,D). Most notably, increasing bulk at the C-terminus decreased β-Arr 2 recruitment efficacies (E_{Max}) at δOR, specifically ~70% for NHEt (**1d**) and $NH^{Cy}Pr$ (**1f**), and 62% for NMe_2 (**1e**). Strikingly, this decrease was even more pronounced at μOR than at δOR **1d** (47%), **1f** (27%), **1e** (26%). Yet larger substituents, such as Pip-4-N(Ph)(CO^nPr) (**1g**), which previously provided a potent and selective analog of Leu-Enk,[36] followed the same trend, and actually delivered an analog with no detectable β-Arr 2 efficacy at μOR (Figure 3D, Table 2). Such decreases in β-Arr 2 efficacy may have beneficial *in vivo* properties, because low arrestin efficacy, especially when paired with partial agonism at the G-protein pathway should provide consistently low *in vivo* adverse effects.[17,19] More so, such low β-Arr 2 efficacy should be preferred relative to calculated bias factors (Table 2), because *in vitro*-determined bias factors are linked to context (e.g. cell and assay systems/endpoints), overvalue the contribution of potency in their calculation, and are difficult to translate to *in vivo* outcomes as bias scores do not factor in pharmacokinetic or pharmacodynamic (particularly ligand residence time) parameters.[37,38] Overall, these structure-function trends clearly indicate that peptides can effectively separate G-protein coupling and β-Arr 2 recruitment at both δOR and μOR through shifts in efficacy, which can facilitate discovery of tool compounds to investigate optimal biased pharmacology for bifunctional δOR/μOR agonists.

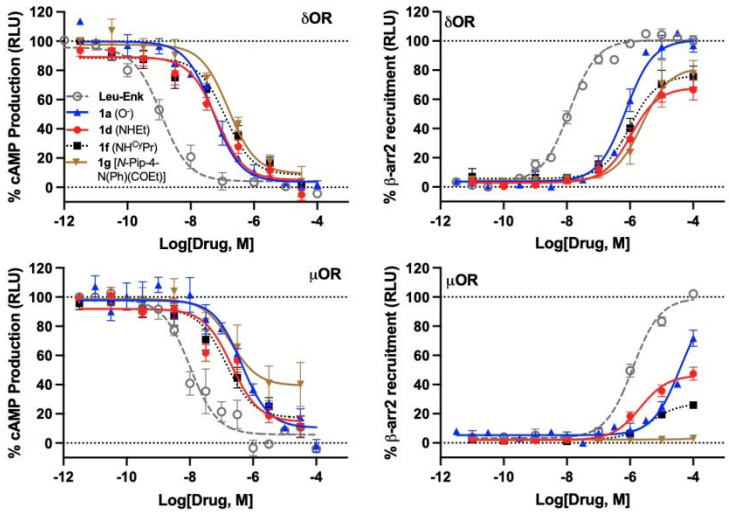


Figure 3. C-Terminal Modifications Delivered δOR agonists with Varying Levels of β-Arr 2 Recruitment at δOR and μOR. (**A**) Inhibition of cAMP Production at δOR; (**B**) β-Arr 2 Recruitment at δOR; (**C**) Inhibition of cAMP Production at μOR; (**D**) β-Arr 2 Recruitment at μOR. Legend: ○ = Leu-Enk (control); ▲ = **1a** (O^-), ● = **1d** (NHEt), ■ = **1f** (NHCyPr), ▼ = **1g** [*N*-Pip-N(Ph)(COEt)]. Each concentration was tested as technical triplicate (cAMP) or duplicate (β-Arr 2), and a minimum of three biological independent replicate dose-response curves were produced for each agonist. Data is normalized to Leu-Enk and for each agonist a composite was produced from the average of the dose response curves of the replicate assays. The error bars depict the standard error of the mean.

Table 2. G-protein Coupling Activities and β-Arr Recruitment Profiles for C-Terminal Analogs of Tyr-ψ[(*Z*)CF=CH]-Gly-Leu-Enk.

Compound	δOR						μOR					
	cAMP pIC_{50}± SEM	cAMP IC_{50} (nM)	β-Arr 2 pEC_{50}± SEM	β-Arr 2 EC_{50} (nM)	β-Arr 2 E_{Max} %±SEM	*Bias Factor**	cAMP pIC_{50}± SEM	cAMP IC_{50} (nM)	β-Arr 2 pEC_{50}± SEM	β-Arr EC_{50} (nM)	β-Arr 2 E_{Max} %+SEM	*Bias Factor**
1a (O^-)	7.47±0.2	33.7	6.12±0.1	764	102±4	1.2	6.40±0.1	363	4.49±0.1	31999	90±10	0.4
1b (NH_2)	7.33±0.1	46.6	6.02±0.1	959	84±14	3.6	6.73±0.1	186	5.58±0.1	2644	92±7	0.2
1c (NHMe)	7.30±0.3	50.2	6.18±0.1	667	90±13	1	7.37±0.2	42.7	5.14±0.2	7215	92±2	0.9

1d (NHEt)	7.18±0.1	66.2	6.16±0.1	695	70±6	3.9	6.75±0.2	178	5.65±0.1	2265	48±5	0.6
1e (NMe_2)	6.37±0.1	425	5.42±0.1	3817	63±7	0.6	6.00±0.2	1000	4.75±0.1	17640	26±4	2.2
1f ($NH^{Cy}Pr$)	6.82±0.2	152	6.16±0.2	693	69±10	1.4	6.90±0.2	126	5.37±0.2	4256	27±1	1.9
1g [Pip-N(Ph)(COEt)]	6.87±0.2	134	5.41±0.1	3926	73±3	1.5	6.74±0.2	183	ND	ND	ND	ND
Leu^5-Enk	8.97±0.1	1.07	7.99±0.1	10.2	100	1	7.70±0.2	20.0	5.89±0.1	1274	100	1

ND = Not Detected. * A bias factor > 1 indicates that a compound is G-protein biased, while a bias factor < 1 indicates that a compound is β-arrestin biased. Except for **1g**, which displayed partial agonism at μOR, all compounds displayed full agonist activity at δOR and μOR.

Modeling at the δOR: The recently published δOR crystal structures in their active-like conformations (peptide-bound: 6PT2 and small molecule-bound: 6PT3)[39] provide a good starting point to better understand possible binding modes of the reported ligands and how they might engage the ligand binding pocket of the δOR. However, first, the relatively low-resolution of the structures as well as the presence of nine thermostabilizing mutations necessitated advanced preparation of the model structures (see SI 1 for details). Nonetheless, with appropriate model optimization, these δOR crystal structures were used to further understand the conformational changes associated with biased agonism at δOR. Molecular modeling was performed using the Schrödinger Suite (Schrödinger, Inc., NY, USA; See SI 1 for details). Molecular docking based on the 2.8Å crystal structure of the peptide agonist-bound δOR (PDB: 6PT2)[39] though the thermostabilizing mutation $D108^{(2.63)}$ was reverted to the WT $K108^{(2.63)}$, as this mutation minimally effected cAMP inhibition and binding affinity of the crystallized peptide, KGCHM07, but decreased β-Arr 2 recruitment.[39] Moreover, based on preliminary modeling (not shown), we predicted that the existence of potential interactions involving the C-terminus with the hydrophobic pocket formed between residues in TM2, TM3 and ECL2, and Phe^4 with residues in ECL3, TM6 and TM7, necessitated the use of the WT $K108^{(2.63)}$. Other thermostabilizing

mutations in the crystal structure were embedded deeper into the orthosteric binding site of δOR near the sodium allosteric binding site, so they were not expected to directly engage in binding interactions with the C-terminus of Leu-Enkephalin analogs.[39] The tyraminium moiety of the peptide agonist KGCHM07[39] was used as a common scaffold for the initial docking of the Leu-Enkephalin analogs (Figure 4A). Additionally, our model retained two crystallized water molecules that maintain a polar network involving Y129$^{(3.33)}$, K214$^{(5.40)}$, H278$^{(6.52)}$, and which enabled the tyraminium portion of the analogs to retain key interactions deep in the binding pocket, while allowing for the flexible docking near the C-terminal modifications of the docked analogs (Figure 4B).

In general, most docked analogs retained a similar alignment of the Gly^2–Gly^3 backbone, featured hydrogen bonding interactions with K108$^{(2.63)}$ and R291^{ECL3}, and engaged in π-stacking interactions between Phe^4 and W284$^{(6.58)}$ (Figures 4B,C, SI-1 Figures 1 & 3). These docked poses were further supported by an all-atom, 200 ns MD simulation using Desmond (see SI-1 for further details). Analog **1a** (O^-) bound to the model δOR structure in a fashion that retained most of the reported interactions with residues K108$^{(2.63)}$, R291$^{(ECL3)}$ and W284$^{(6.58)}$ (Figure SI-5).[39] However, the Phe^4 residue of analog **1a** (O^-) formed a C_{Aryl}-H interaction with W284$^{(6.58)}$ instead of the expected π-π interaction largely due to the insufficient rotation of the side chain of W284$^{(6.58)}$ (Figure 4B).[39] Analogs **1b**–**1g** showed π-stacking interactions for which the rotation of the W284$^{(6.58)}$ side chain enabled the benzyl moiety to access a hydrophobic pocket between TM6, TM7 and ECL3 (SI-1 Figures SI-1 & SI-3). Furthermore, Leu-Enkephalin analog **1a** (O^-) was embedded further into the binding pocket relative to the crystallized peptide, KGCHM07, while forming two π-π interactions with W274$^{(6.48)}$ and Y308$^{(7.42)}$, possibly due to the presence of the thermostabilizing mutation S131$^{(3.35)}$ in the sodium binding site (Figure SI-6). Near the C-

terminus of compound **1a**, R291^{ECL3} appeared to facilitate an interaction between Phe^{4} and W284$^{(6.58)}$ via a π-π interaction, as well as a water-mediated H-bond with C198$^{(45.50)}$. Future studies using optimized and dynamic model structures of the δOR will further deduce the role mediated by the C-terminal modifications of peptide agonists at δOR.

Based on our docking model at the δOR, C-terminal modifications of peptide agonists may underrecruit β-Arr 2 through two potential interactions. First, in the C-terminal groups of the docked analogs mainly interacted with K108$^{(2.63)}$, W284$^{(6.58)}$, R291^{ECL3}. Of the three residues, R291^{ECL3} has been reported to act as a mediator for peptide selectivity at opioid receptors, by helping position W284$^{(6.58)}$ to engage naltrindole with a π-π interaction.[40] Similarly, modifications of the C-termini of analogs **1a**–**1g** modulated the interactions of peptide agonists with K108$^{(2.63)}$/W284$^{(6.58)}$/R291^{ECL3}, and specifically, we speculate that the perturbation of this interaction network may induce conformational changes in TM6/7 and ECL3 with implications on arrestin recruitment, through biophysical and pharmacological experiments to probe this hypothesis are beyond the scope of the present manuscript. Second, the Leu^{5} side chain fits within a narrow hydrophobic pocket in the δOR involving K108$^{(2.63)}$ and W114$^{(23.50)}$, and in this region, analogs with decreased β-Arr 2 efficacies have poor overlap with the docked pose of **1a** (Figure 4C). We hypothesize that the different orientations of the Leu^{5} side chain might arise from increased steric bulk at the C-terminus that pushes the side chain out of its energetically favorable orientation, which is also supported by previous studies in which substitution of the Leu^{5} side chain also modulates β-Arr 2 efficacy.[34]

Modeling at the μOR: Further modeling of peptide-μOR interactions using morphinan agonist BU72 bound 2.1 Å mouse μOR crystal structure (PDB: 5C1M)[41] provided possible interactions that could rationalize the decreased β-Arr 2 recruitment efficacy imparted by C-

terminal modifications (Figure 4D; See SI for details on modeling methodology). However, the flexibility of the docked peptides in this model, especially in the presence of the crystallized water molecules, could be addressed in future studies using dynamic structures to further probe key interactions. In the docked pose of compound **1a,** the ligand engages multiple residues on TM3, while the C-terminal carboxylate engages both K233$^{(5.40)}$ and K303$^{(6.58)}$ in favorable charged interactions (Figures 4E). Near the C terminus, the Phe^{4} side chain resides in a hydrophobic pocket composed of L219^{ECL2} and F221^{ECL2} and the Leu^{5} side chain presents towards solvent. Using enhanced sampling modeling of analogs **1a**–**g**, the conversion of the ligand's charged C-terminus to neutral amides resulted in similar binding poses, with different interactions with between the Leu^{5}-amide and K303$^{(6.58)}$ (Figure 4F, also See SI 1). Notably, though some C-terminal amides interact with K303$^{(6.58)}$, none of the analogs engage the TM2/ECL3 region of the receptor, which make key interactions in the docked models of the δOR. Moreover, no systematic major conformational differences within the binding pocket or rotational differences around the Phe-piperidine amide bond correlated with changes to β-Arr 2 or cAMP efficacies.[42–44]

Considering this model, the interactions between the ligand's C-terminus and K233$^{(5.40)}$ and K303$^{(6.58)}$ might be critical to modulating β-Arr 2 recruitment, as molecular modeling of a macrocyclic peptide with strong β-Arr 2 recruitment was shown to move TM5 and TM6 inward, whereas G-protein biased peptides did not encourage an inward movement of these μOR helices.[43] Further, though several ligands, including BU72, morphine, DAMGO, and fentanyl make strong interactions with D147$^{(3.32)}$, Y148$^{(3.33)}$, and Y326$^{(7.43)}$,[42,43,45–47] these interactions don't seem to correlate with ligand bias. Hence, the design of peptide agonists that gain affinity

from interactions with TM3, TM7, but that perturb the positioning of TM5 and TM6 could lead to the modulation of β-arrestin bias.

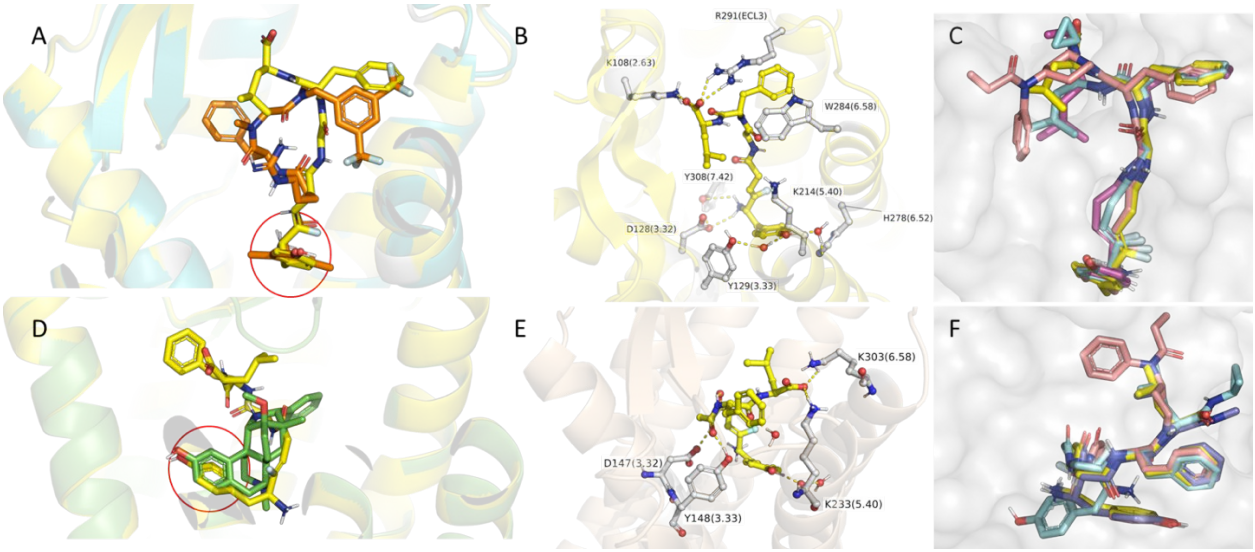


Figure 4. Molecular Docking into a Model Based on δOR (PDB: 6PT2) and μOR (PDB: 5C1M). **(A)** Selected docked pose of analog **1a** (yellow) aligned on the crystal pose of KGCHM07 (orange); PDB 6PT2 crystal structure (cyan ribon) and docked structure starting with 6PT2 (yellow ribbons). The red circle encompasses the common scaffold selected for initial docking. **(B)** At the C-terminal, analog **1a** (O^-) forms hydrogen bonds with $K108^{(2.63)}$ and $R291^{(ECL3)}$ and C_{Aryl}–H-π interaction with $W284^{(6.58)}$. $R291^{(ECL3)}$ appears to mediate the key interactions between the C-termini of the Leu-Enk analogs and δOR. **(C)** Larger C-terminal substituents push the respective Leu^5 side chains or *N*-Pip-N(Ph) moiety further into a narrow hydrophobic pocket. Notably, for **1h**, the N(Ph) group presents toward $L200^{(45.52)}$ and $F202^{(ECL2)}$ (the pocket in which the Leu^5 side chains reside) and the COEt group presents towards unoccupied space and does not make constructive interactions with the receptor. **(D)** Selected docked pose of analog **1a** aligned on the crystal pose of BU70 (green), PDB 5C1M crystal structure (green). and docked structure starting with 5C1M (yellow ribbons). The red circle encompasses the common scaffold selected

for initial docking. **(E)** In the μOR model, analog **1a** forms a hydrogen bond with K303$^{(6.58)}$, but does not appear to interact with N127$^{(2.63)}$ or with residues in ECL3. Furthermore, the pocket is more open near the C-terminal region and the Leu^5 side chain of the analogs. **(F)** Aligned poses of analogs docked into μOR. See SI for more details. *Compounds Depicted:* **1a** (O^-) yellow, **1d** (NHEt) purple, **1f** ($NH^{Cy}Pr$) cyan, **1g** [*N*-Pip-N(Ph)(COEt)] pink. Receptor side chains in panels B, E are depicted in white.

Conclusion

Overall, the experimental data and computational modelling identify the Leu^5 C-terminus of Leu-Enk as a key site to regulate β-Arr 2 recruitment through both the δOR and μOR, which provides an important benchmark for μ/δOR agonists for which data for β-Arr 2 recruitment is generally unavailable. Nonetheless, no previous analogs have been reported that display decreased efficacy at β-Arr 2 at both the δOR and μOR, and thus future *in vivo* characterization of improved Leu^5 analogs will provide broader understanding of how biased signaling at μOR/δOR cooperatively impact nociception and side effect profiles. Considering the excellent stability imparted by the Tyr-ψ[(Z)CF=CH]-Gly substitution[29] (See SI), these C-terminal substituted Leu-Enk analogs provide excellent leads for further optimization to deliver biased ligands for the δOR for treating pain. By combining such C-terminal modifications with other structural modifications that improve δOR/μOR potency and/or selectivity, it should be possible to develop a range of tool compounds for thoroughly investigating δOR/μOR dual agonists and δOR-selective agonists with low β-Arr recruitment efficacies. Testing such future analogs with well-defined β-Arr profiles side-by-side in models of chronic pain, particularly in a design that includes repeated administration, may help validate the utility of metabolically stabile signal-biased δOR/μOR agonists.

EXPERIMENTAL PROCEDURES

Synthesis of Peptides: Analogs were synthesized using a microwave synthesizer using a solution-phase protocol using Boc chemistry and 4N HCl in 1,4-dioxane for deprotection.[35] Purification and determination of the purity of final compounds was conducted using reversed phase chromatography using appropriate gradients.

Pharmacological Characterization: As previously described,[27] we assessed binding affinity using a competition radioligand binding assay, G protein potency and efficacy using a cAMP GloSensor assay, and β-Arr 2 recruitment via PathHunter assays at both δOR and μOR, using Leu-Enk as the reference compound. A minimum of three independent values were obtained for each compound in each of the cellular assays. Bias factors were calculated using the operational model equation in Prism 8 [Log R (τ/KA)] as previously described[48,49] using Leu^5-Enk as reference compound.

Conflict of Interests

The authors declare no competing financial interests.

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