Title: Modulating β Arrestin-2 Recruitment at the δ- and μ-Opioid Receptors

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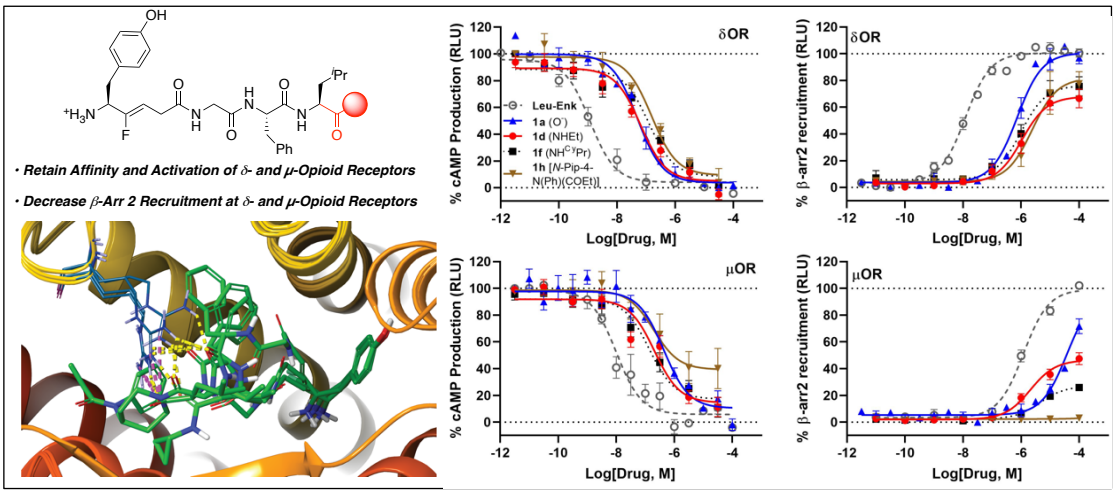
Key words: Leu-enkephalin; delta opioid receptor; mu opioid receptor; beta-arrestin; biased signaling;

TOC Figure



Abstract

Opioid receptors can trigger two distinct pathways (G protein coupling and arrestin recruitment) that differentially regulate a host of desired and undesired pharmacological effects. Increasingly, "biased" opioids that selectively activate one pathway over the other are being developed to treat disorders in which μ- and κ-opioids receptors are involved, though the development of biased δ-opioid receptor agonists has remained rather quiescent. Herein, we identify the C-terminus of Tyr-ψ[(*Z*)CF=CH]-Gly-Leu-enkephalin as a key site to regulate bias of both δ- and μ-opioid receptor agonists. Using *in vitro* assays, substitution of the Leu^5 carboxylate reduced β-arrestin recruitment through both the δ- and μ-opioid receptors in a predictable structure-dependent fashion, while retaining affinity and cAMP potency comparable to the C-terminal carboxylate. These substitutions should enable discovery of a range of tool compounds for exploring δ-opioid receptor pharmacology and toxicology, which will enable reevaluation of this target within the context of biased signaling.

Introduction

Activation of μ-opioid receptor (μOR) provides rapid and potent pain relief, and as such μ-opioids remain first line treatment for perioperative pain management. Outside of palliative care, prolonged use of such opioids causes problematic adverse effects, including the development of tolerance, opioid-induced constipation, and respiratory depression, which limit their utility for treating non-acute pain.[1] Due to these side effects, chronic pain management guidelines restrict currently available μOR drugs to 2nd or 3rd line treatments.[2,3] To extend the utility of μOR agonist analgesics to include treatment of chronic pain disorders, G protein-biased μOR agonists, those that selectively signal through cyclic-AMP (cAMP) pathways as opposed to β-arrestin (β-Arr) pathways, are proposed to reduce the opioid side effect profile, whilst retaining analgesic potency.[4] Though G-protein-biased μOR agonists, such as TRV130 and PZM21, appeared to be promising candidates,[5,6] recent data challenges the premise that these biased μOR analgesics are indeed superior to current clinically used μOR analgesics, particularly with respect to (1) the development of opioid-induced hyperalgesia upon prolonged use, and (2) whether these opioids can reduce the negative symptoms observed in patients with chronic pain.[7–10] In contrast to μORs, δ-opioid receptors (δORs), have the potential to treat chronic pain, including inflammatory pain, neuropathic pain and migraine, but are not associated with the μOR-related adverse effect profile.[11–14] At present, no δOR-selective opioid agonists have been approved for clinical use, in part due to δOR agonists causing seizures,[15] a side effect that has been associated with β-Arr 2 recruitment.[16,17]

Because of the described limitations associated with μOR-selective and δOR-selective opioids, a pharmacological strategy for treating acute and chronic pain has emerged that relies on dual activation of both the μOR and δOR,[18–27] and some of these bifunctional agonists indeed

display antinociception with reduced tolerance, dependence, locomotor activation and self-administration relative to classical morphinans.[18–20,27] Thus far, the development of these μOR/δOR dual agonists has largely ignored β-Arr 2 recruitment, which makes it impossible to correlate their reduced side effect profile with μOR/δOR dual agonism as opposed to β-Arr 2 recruitment. In a single example, 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.[22] Thus, μOR/δOR dual agonists with well-characterized β-Arr 2 profiles are essential to validate μOR/δOR dual agonism as a desired pharmacological profile.

We recently showed that small modifications of Phe^4 of Leu^5-enkephalin (Leu-Enk, YGGFL), an endogenous opioid peptide activating δORs, can alter arrestin recruitment,[28] while other δOR pentapeptides exist that display biased signaling profiles.[29] As such, derivatization of δOR peptides can facilitate the study of biased-signaling in relation to desired δOR-mediated antinociception and undesired adverse effects. Herein, we derivatize the carboxyl-terminal region of previously reported Leu^5-Enk pepidomimetics[30,31] with the goal of delivering a set of opioid peptides with varying degrees of β-Arr recruitment, in particular with limited μOR β-Arr recruitment, as such compounds remain unidentified. Further, computational modeling points to key ligand-target interactions that regulate β-Arr 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.

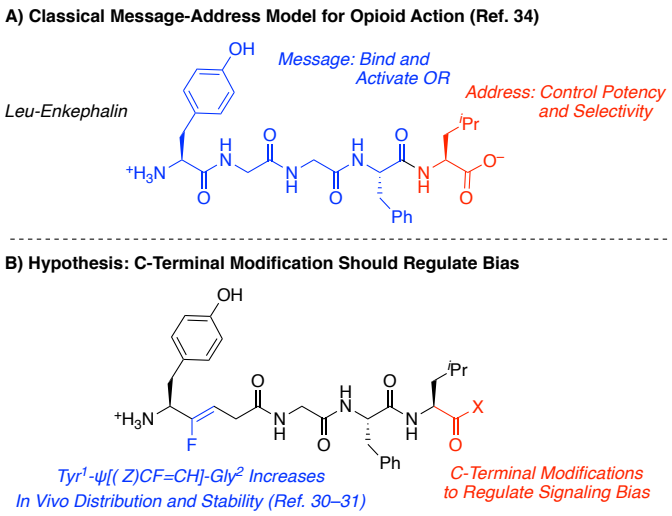


Figure 1. Designing Leu-Enk Analogs with Decreased β-Arr 2 Recruitment. (A) In the classical "Message-Address" model for opioid action, C-terminal modifications might regulate biased signaling at the δOR (cAMP vs. β-Arr 2). (B) The present work exploits C-terminal modifications in the "Address" domain 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, an endogenous peptide that acts at the δOR with 1–5-fold binding affinity over μOR, and >1000-fold over κOR,[32,33] 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.[34] 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 encodes the required properties to recognize and bind 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).[34] Though this hypothesis was developed prior to recognition of opioid-induced β-Arr

signaling, we speculate that the message-address concept might translate to the development of biased ligands, specifically that C-terminal modifications of Leu^5-Enk might be designed to improve δOR selectivity over μOR as well as reduce β-Arr recruitment potency at μOR (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,[35] 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 (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).[30,31]

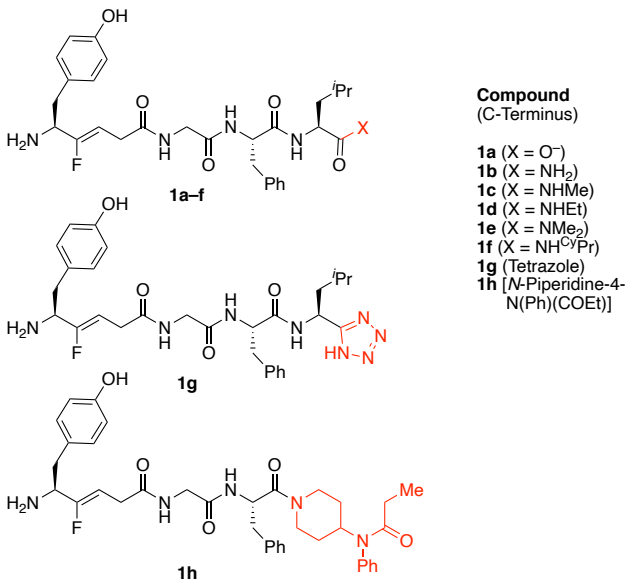
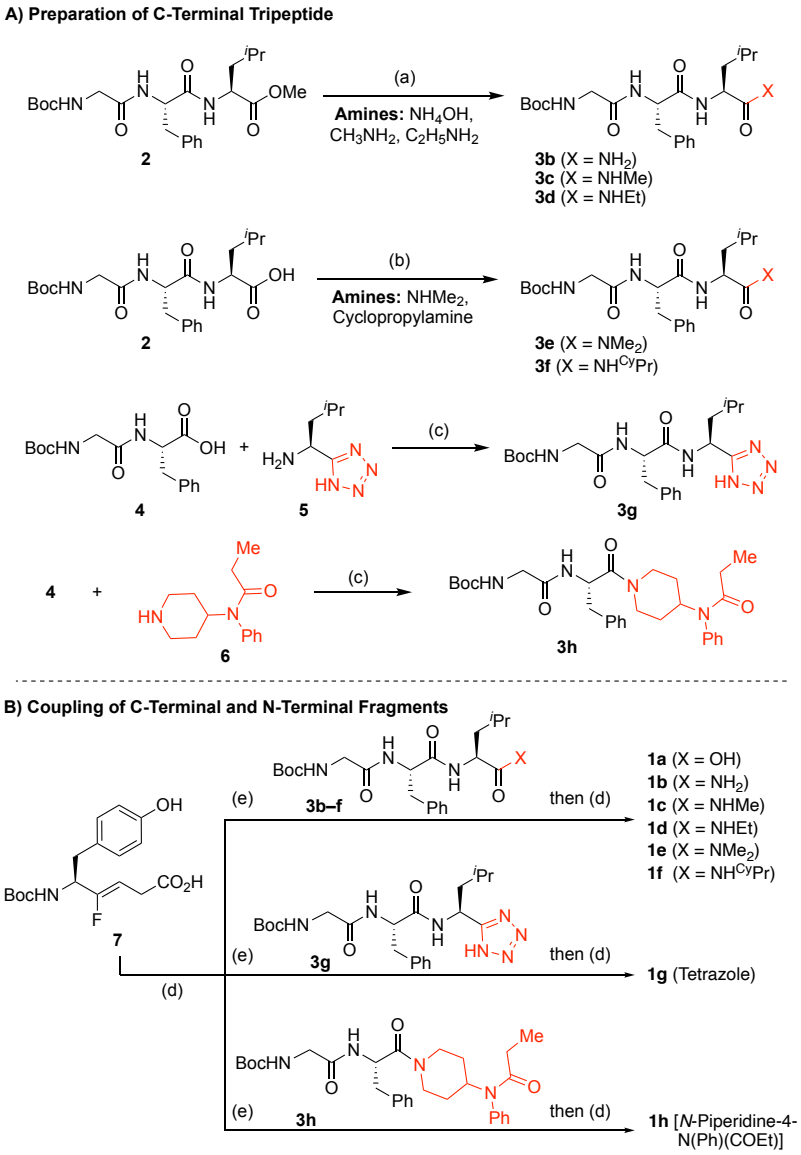


Figure 2. C-Terminal Substitutions Synthesized and Pharmacologically Characterized.

Synthesis of Analogs: Analogs were prepared using microwave-assisted solution phase coupling chemistry according to a Boc-protection strategy (Scheme 1).[36] C-terminal functionalized

tripeptides were accessed from the corresponding methyl esters via (a) heating with a mixture of amine : MeOH (X = NH_2, NHMe, NHEt), or from the corresponding acids via (b) coupling with DIC/HONB under microwave irradiation (X = NMe_2, $NH^{Cy}Pr$), or (c) coupling of the C-terminal moiety with Boc–Gly–Phe–OH using DIC/HONB under microwave (MW) irradiation [tetrazole, *N*-Piperidine-4-N(Ph)(COEt); Scheme 1A]. These tripeptides were deprotected using HCl in 1,4-dioxane, then coupled onto our previously reported Tyr-ψ[(*Z*)CF=CH]-Gly–OH dipeptidemimetic[30,31] using DIC/HONB/DIEA under microwave irradiation and subsequently deprotected (Scheme 1B). Purification by reverse phase HPLC provided analytically pure samples for pharmacological evaluation.



Scheme 1. Synthesis of Analogs **1a**–**h**. 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).

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 (Tetrazole)	7.46±0.1	34.8	7.38±0.1	42.1	1.2
1h [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

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

Using a standard competition radioligand binding assay and [^{3}H]DPDPE or [^{3}H]DAMGO as control compounds, C-terminal substituted analogs **1b**–**g** engaged both the δOR and μOR within an order of magnitude of parent compound **1a**, with bulky analog **1h** 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, $NH^{Cy}Pr$ and Tetrazole; **1b–d**, **f–g**) possessing better binding affinities relative to analogs bearing bulky NMe_2 and Pip-N(Ph)(COEt) (**1e**, **1h**) substituents. Further, analogs **1b–1f** bearing C-terminal amides (NH_2, NHMe, NHEt, NMe_2, $NH^{Cy}Pr$) 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 analogs **1g–h** [tetrazole, Pip-N(Ph)(COEt)] that bound to the two receptors with equal affinities (1.0–1.2).

Despite these binding trends, analogs **1b**–**h** 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.[28] 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 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)($CO^{n}Pr$), which previously provided a potent and selective analog of Leu-Enk,[37] followed the same trend, and actually delivered an analog with no detectable β-Arr 2 efficacy at μOR (Figure 3D, Table 2, entry 1h). Such decreases in β-Arr 2 efficacy should 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.[38] More so, such low efficacy β-Arr 2 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 potency, and are difficult to translate to *in vivo* outcomes due to issues with pharmacokinetic/pharmacodynamic relationships. 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 help attenuate β-Arr 2 recruitment and likely the subsequent side effect profiles.

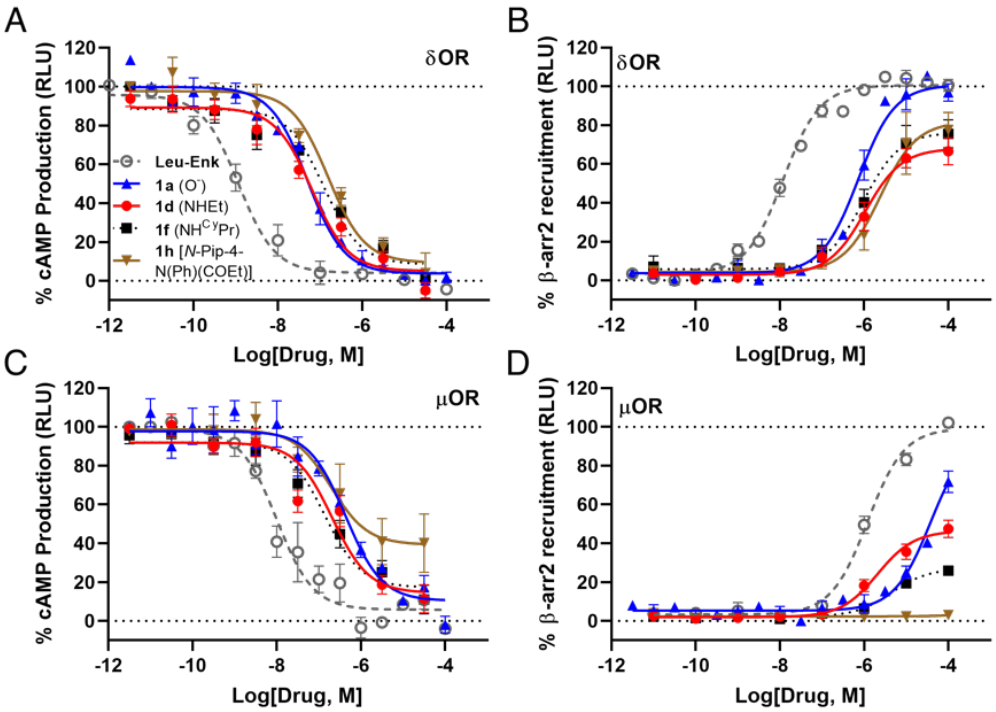


Figure 3. C-Terminal Modifications Delivered Potent δOR agonists with Varying Levels of β-Arr 2 Recruitment at δOR and μOR. (**A**) Inhibition of cAMP Production at δOR; (**B**) β-Arr 2 Recruitment at δOR; (**C**) Inhibition of cAMP Production at μOR; (**D**) β-Arr 2 Recruitment at μOR.

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 Efficacy %±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 Efficacy %+SEM	*Bias Factor*
1a (O^-)	7.28±0.2	53.1	6.12±0.1	764	102±4	1.2	6.44±0.1	364	4.49±0.1	31999	90.2±10	0.4
1b (NH_2)	7.33±0.1	46.6	6.02±0.1	959	83.6±14	3.6	6.73±0.1	187	5.58±0.1	2644	92.1±7	0.2
1c (NHMe)	7.30±0.3	50.2	6.18±0.1	667	89.7±13	1	7.37±0.2	42.6	5.14±0.2	7215	92.4±2	0.9
1d (NHEt)	7.18±0.1	66.2	6.16±0.1	695	70.4±6	3.9	6.75±0.2	177	5.65±0.1	2265	47.7±5	0.6
1e (NMe_2)	6.37±0.1	425	5.42±0.1	3817	62.5±7	0.6	6.00±0.2	999	4.75±0.1	17640	25.6±4	2.2
1f ($NH^{Cy}Pr$)	6.82±0.2	152	6.16±0.2	693	69.4±10	1.4	6.90±0.2	126	5.37±0.2	4256	27.0±1	1.9
1g (Tetrazole)	7.52±0.1	30.5	6.31±0.1	485	85.2±2	3.5	6.20±0.2	633	5.1±0.2	7893	55.1±9	0.5
1h [Pip-N(Ph)(COEt)]	6.87±0.2	134	5.41±0.1	3926	73.1±3	1.5	6.90±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	19.9	5.89±0.1	1274	100	1

Table 2. G-protein Coupling Activities and β-Arr Recruitment Profiles for C-Terminal Analogs of Tyr-ψ[(*Z*)CF=CH]-Gly-Leu-Enk. ND = Not Detected

Modeling: Computational modeling based on the recently published structure of the δOR bound to a peptide agonist (PDB ID 6PT2)[39] provided insight into the key ligand-δOR interactions that regulate bias. Modeling was performed using rigid-receptor, pharmacophore-constrained docking via Glide[40–42] followed by full atom refinement of the interaction site using Prime[43,44] for all analogs. Based on this model of the δOR, C-terminal modifications may underrecruit β-Arr 2 though two distinct interactions. First, in the docked poses, transmembrane (**TM**) domains 6/7 and extracellular loop (**ECL**) 3 adopt distinct conformations (Figure 4A), and we speculate that perturbation of these helices causes intracellular distortion that in turn affects β-Arr 2 recruitment. In the docked structures, the other TM helices showed minimal, if any signs of distortion, though many of the residues were free to reorient. In these poses, a critical interaction exists between the C-terminal group (acid or amide) and R291 on ECL3, which is a critical for imparting δOR selectivity,[39] and we speculate that disruption of this interaction may cause the disorder in TM6/7 and ECL3 (Figure 4B). Second, the Leu^5 side chain fits within a narrow hydrophobic pocket in the δOR, and in this region, analogs with decreased β-Arr 2 efficacy have poor overlap with the acid's pose (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 normal orientation, which is also supported by previous studies in which substitution of the Leu^5 side chain also perturbs β-Arr 2 efficacy.[35]

Further modeling of ligand-μOR interactions using a morphinan agonist bound μOR (PDB ID 5C1M)[45] also help rationalize the decreased β-Arr 2 recruitment efficacy imparted by C-terminal modifications. Of note, there are no significant constructive interactions between TM6/7 or the EL, likely due to the lack of constructive interactions involving TM7 and the EL. In this case, the

docking model of **1a** enables the C-terminal carboxylate to engage both Lys303 (TM6) and Lys 233 (TM5) in favorable charged interactions (Figure 4D), of which only Lys303 dictates how the ligand will interact with this key region of the receptor that might regulate the β-Arr 2 recruitment. Using enhanced sampling modeling of analogs **1a**–**h**, removal of the charge results in similar binding poses, with different interactions with Lys303 (representative examples in Figure 4E–F). Notably, though some C-terminal amides make interactions with Lys303, which affects affinity to the μOR, none of the analogs engage the TM7/ECL3 region of the receptor, which presumably increases disorder in the intracellular domain that is relevant for β-Arr 2 recruitment. Overall, these data and models support the C-terminal region of Leu-Enk as a key site for perturbing β-Arr 2 recruitment at both the δOR and μOR.

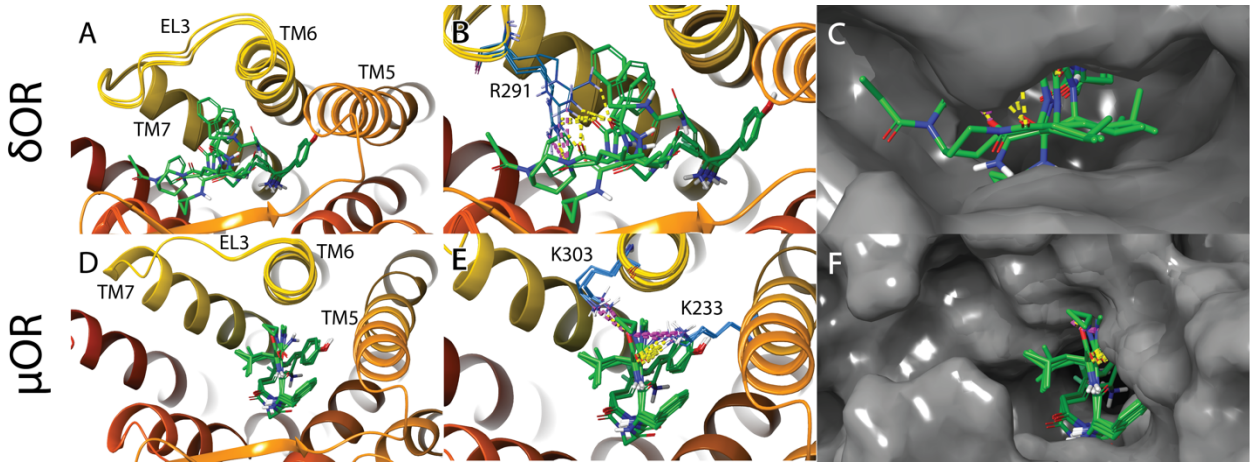


Figure 4. Computational Dockings into the δOR (PDB: 6PT2) and μOR (PDB: 5C1M) suggests that (**A**) analogs perturb the conformations of the δOR's TM6/7 and ECL3 (yellow), and that (**B**) the on the δOR's R291 on ECL3 makes a key contact with the C-terminal group of different analogs. Further, (**C**) C-terminal modification pushes the Leu^5 side chain into different conformations in a narrow hydrophobic pocket in the δOR. In contrast, (**D**) analogs do not perturb the conformations of TM6/7 and ECL3 (yellow) in the μOR, (**E**) K303 on TM6 (yellow)

and K233 on TM5 of the μOR make key contacts with the C-terminal group of different analogs, and (**F**) the μOR pocket is much more open near the C-terminal region of the analogs. Based on these models, we speculate that perturbation of the C-terminal functional group and/or increasing steric bulk around Leu^5 will further perturb β-Arr 2 recruitment. *Compounds Depicted:* YGGFL–CO_2^-, YGGFL–NHEt, YGGFL–$NH^{Cy}Pr$, YGGFL–Pip–N(Ph)(COEt)

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 contrasts other μ/δOR agonists in the field for which data for β-Arr 2 recruitment is unavailable. Nonetheless, no previous analogs have been reported that underrecruit β-Arr 2 at both the δOR and μOR, which should provide antinociception with decreased adverse effect profiles. Considering the excellent stability imparted by the Tyr-ψ[(Z)CF=CH]-Gly substitution[30] (See Supporting Information), 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, δOR/μOR antagonists, and δOR-selective agonists with low β-Arr recruitment efficacy. Testing such future analogs with well-defined β-Arr profiles side-by-side in a model of chronic pain, particularly in a design that includes repeated administration, would provide novel insight into the treatment of chronic pain while minimizing adverse effects.

EXPERIMENTAL PROCEDURES

Solution Phase Synthesis of Peptides: Peptidomimetics were synthesized using a Biotage Initiator microwave synthesizer using a solution-phase peptide synthesis protocol using Boc chemistry and 4N HCl in 1,4-dioxane for deprotection.[36] Purification was conducted using a Teledyne ISCO EZ Prep system with a RediSep® C18 Prep column (30x250 mm, 100 Å). Purity analysis of final tested compounds was carried out using a Waters UPLC Aquity system equipped with a PDA eλ detector (200 to 400 nm) and a HSS T3 C18 column, (1.8 μM, 2.1x50 mm column), using one of two methods. Protocol A: gradient elution of 2% MeCN / 98% H_2O (with 0.1% formic acid) to 98% MeCN over 2.5 min, then holding at 98% MeCN for 1 min at a flow rate of 0.7 mL/min at 40 °C. Protocol B: gradient elution of 2% MeCN / 98% H_2O (with 0.1% formic acid) to 98% MeCN over 2.5 min, then holding at 98% MeCN for 3 min at a flow rate of 0.8 mL/min.

Pharmacological Characterization: To characterize our substituted analogs, we assessed binding affinity by competition radioligand binding, 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 reference compounds as previously described in full detail.[28]

Data and Statistical Analysis: All data are presented as means ± standard error of the mean, and analysis was performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA). For in vitro assays, nonlinear regression was conducted to determine pIC_{50} (cAMP) or pEC_{50} (β-Arr 2 recruitment). Technical replicates were used to ensure the reliability of single values, specifically each data point for binding and β-Arr recruitment was run in duplicate, and for the cAMP assay in triplicate. The averages of each independent run were counted as a single experiment and combined to provide a composite curve. In each experimental run, Leu-Enk was utilized as a positive control/reference compound to allow the data to be normalized and to

calculate the log bias value. A minimum of three independent values were obtained for each compound in each of the cellular assays.

Calculation of Bias Factor: Bias factors were calculated using the operational model equation in Prism 8 to calculate Log R (τ/KA) as previously described.[46] Subsequently, bias factors were calculated using Leu^5-Enk as reference compound. A bias factor > 1 meant that the agonist was more G protein-biased than the reference compound; A bias factor < 1 meant that the agonist was less G protein-biased than the reference compound.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XX

Experimental details for the synthesis of, characterization of, and determination of purity for compounds **1b–1h**, detailed pharmacological procedures, additional pharmacological characterization, computational procedures, stability data.

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K.K.S. design, synthesis, and characterization of analogs, analysis of data, outlining of manuscript. R.J.C. data acquisition and analysis, supervision and original draft preparation. H.S., A.B.T., B.R.C., and K.L.M. data acquisition and analysis. D.J. computational modelling and visualizations, draft preparation. R.M.vR.: design and conceptualization, data analysis, original draft preparation, funding acquisition and supervision. R.A.A. design and conceptualization, data analysis, original draft preparation, funding acquisition and supervision. All authors proofread and approved the final draft.

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Notes

The authors declare no competing financial interests.

ABBREVIATIONS

β-Arr 2, beta-arrestin 2; cAMP, 3',5'-Cyclic adenosine monophosphate; CNS, Central nervous system; DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin; DIC, *N,N'*-Diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; δOR, Delta opioid receptors; DMF, *N,N*-Dimethylformamide; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; ECL, Extracellular loop; HONB, *N*-Hydroxy-5-norbornene-2,3-dicarboxylic acid imide; μOR, Mu opioid receptors; MW, Microwave; Leu-Enk, Leu-Enkephalin; TM, Transmembrane; UPLC, Ultra performance liquid chromatography.

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