Structural basis for bifunctional peptide recognition at human δ-opioid receptor

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Bifunctional µ- and δ-opioid receptor (OR) ligands are potential therapeutic alternatives, with diminished side effects, to alkaloid opiate analgesics. We solved the structure of human δ-OR bound to the bifunctional δ-OR antagonist and µ-OR agonist tetrapeptide H-Dmt-Tic-Phe-Phe-NH₂ (DIPP-NH₂) by serial femtosecond crystallography, revealing a cis-peptide bond between H-Dmt and Tic. The observed receptor-peptide interactions are critical for understanding of the pharmacological profiles of opioid peptides and for development of improved analgesics.

The management of pain, mood states and other neurophysiological processes is regulated by the release of classical endogenous opioid peptides, such as endomorphins, enkephalins and dynorphins, that selectively bind to and activate their respective µ-, δ- and κ-OR subtypes1. Alkaloid opiate such as morphine, targeting µ-OR, are the most widely used analgesics for the treatment of moderate to severe pain, but chronic administration produces side effects such as tolerance, dependence and addiction. Coadministration of the δ-OR antagonist naltrindole has been shown to prevent the development of morphine-induced tolerance and dependence2, thus prompting the design of compounds with a mixed δ-OR–antagonist and µ-OR–agonist function. This bifunctional pharmacological profile has been achieved with both morphinan-based small molecules and opioid-peptide analogs, to produce compounds with reduced liability for tolerance and dependence in vivo, thus suggesting their high therapeutic potential3,4.

The bifunctional δ-OR–antagonist and µ-OR–agonist tetrapeptide DIPP-NH₂ (H-Dmt-Tic-Phe-Phe-NH₂, with Dmt representing 2,6-dimethyltyrosine and Tic representing 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) (Fig. 1) is a member of the so-called H-Tyr-Tic-Phe-Phe-OH (TIPP) class of endomorphin-derived peptide analogs displaying profiles of δ-OR–antagonist activity or mixed δ-OR and µ-OR activity5–7. Subtle changes in their chemical structure were previously found to modulate the functional profiles of these ligands8,9. The most noteworthy modulation was achieved by replacement of a proline (present in endogenous peptides such as endomorphin-2, H-Tyr-Pro-Phe-NH₂) by a Tic scaffold, to result in potent compounds with mixed δ-OR–antagonist and µ-OR–agonist activities10, including DIPP-NH₂ (ref. 7). However, the structural basis leading to these pharmacological profiles is not understood.

To gain structural insights into the binding mode and OR-subtype specificity of DIPP-NH₂, we engineered and crystallized a receptor construct containing the thermostabilized apocytochrome bsG2RIL (BRIL) fused to the N terminus of human δ-OR (residues 38–336, BRILΔNδ-OR) in complex with DIPP-NH₂ (Supplementary Fig. 1 and Online Methods). Radioligand competition data confirmed that the construct used for structure determination binds DIPP-NH₂ with similar affinity as that of the wild-type (WT) receptor (Supplementary Fig. 2). We initially determined the X-ray crystal structure of the BRILΔNδ-OR–DIPP-NH₂ complex at 3.3-Å resolution, using synchrotron X-ray diffraction of cryocooled crystals. Subsequently, we applied a recently developed serial femtosecond crystallography approach in lipidic cubic phase (LCP)11,12, using an X-ray free electron laser (XFEL), and determined the room-temperature structure of the complex at 2.7-Å resolution.

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Figure 1: Structure of the BRILδ-OR-DIPP-NH₂ complex. (a) Overall view of δ-OR (purple cartoon, with ECL2 in red) in complex with DIPP-NH₂ (orange sticks and transparent spheres); residues lining the binding pocket are shown as light-blue sticks, hydrogen bonds as black dashed lines, and water molecules as red spheres. (b) Chemical structures of DIPP-NH₂, endomorphin-1 and endomorphin-2 showing the structural similarities between the peptide analog DIPP-NH₂ and endogenous OR peptides. (c) Close-up view of the DIPP-NH₂-binding site, residues forming the DIPP-NH₂ pocket are shown as light-blue sticks. (d) Sliced surface representation of the peptide-binding pocket. The omit Fᵋ – Fᵋ electron density around the peptide DIPP-NH₂ is contoured at 3σ and shown as a blue mesh.

(Fig. 1, Supplementary Figs. 3 and 4 and Supplementary Table 1). Despite subtle differences, both BRILδ-OR-DIPP-NH₂ structures are very similar, with a r.m.s. deviation (r.m.s.d.) of 0.5 Å over all structurally characterized receptor Cα atoms, and therefore we used the higher-resolution XFEL structure in subsequent analysis. Overall, the inactive-state δ-OR–DIPP-NH₂ structure is similar to the previously determined 1.8-Å-resolution structure of δ-OR bound to the morphinan derivative naltrindole13 (r.m.s.d. of 0.85 Å, excluding the plane of the Dmt phenol ring deviates approximately 2 Å of the extracellular loop 2 (ECL2) (∆r = 2 Å), Phe4-NH₂ is less restricted, with cis configuration with the Tic side chain in the gauche− conformation, overlaying with the benzene moiety of the indole ring in naltrindole (Fig. 2d)13. The observed binding pose of DIPP-NH₂ is consistent with NMR spectroscopic data showing that TIPP peptides in solution undergo a slow dynamic exchange between conformations containing cis and trans configurations of the Tyr1-Tic peptide bond17. Similarly, a cis configuration of the Tyr1-Pro2 amide bond was also proposed as the bioactive conformation in endomorphin analogs18. The Tic side chain occupies a hydrophobic pocket formed by helices VI and VII, adjacent to that occupied by Dmt. This pocket is formed by the side chains of Ile2776.51, Ile3047.39, Leu3067.35, Trp2846.58 and Val2816.55, with the aromatic group of Tic making a π–π interaction with Trp2846.58 and stacking with the Val2816.55 side chain (Fig. 1). The interactions of Tic and the Dmt 2′ methyl group with Val2816.55 apparently contributes to an ~1.1-Å outward shift of the Val2816.55 side chain on the extracellular side of helix VI, as compared to the naltrindole-bound δ-OR structure13 (Fig. 2d).

The δ-OR-DIPP-NH₂ structure highlights important atomic details for the bifunctional pharmacological profile of DIPP-NH₂ at the µ- and δ-OR, which is centered prominently around the pocket binding the Tic chemotype. Superposition of the current δ-OR–DIPP-NH₂ structure with the µ-OR inactive-state structure (PDB 4DKL)15 reveals that the Tic pharmacophore clashes with side chains of nonconserved Trp3187.35 and Lys3036.58 in the µ-OR (equivalent to Leu3067.35 and Trp2846.58 in δ-OR, respectively) (Fig. 2a,b).
The δ-OR double mutant, bearing mutations L300^W and W284^K, demonstrated a decrease of over two orders of magnitude in the affinity of both H-Tyr-Ala-Gly-Phe-Leu-OH (DADLE) and DIPP-NH₂ peptides (data not shown), thus preventing further characterization of the functional effects of these mutations. Because Tic is critical for the bifunctional profile, this divergent interaction site is likely to have a key role in defining δ-OR–agonist versus δ-OR–antagonist properties of opioid peptide ligands. DIPP-NH₂ had previously been characterized as a δ-OR antagonist and μ-OR agonist in the classical mouse vas deferens and guinea pig ileum functional assays. The present pharmacological data obtained in cell-based assays confirmed that the peptide is a full agonist at the μ-OR, with similar potency and efficacy as those of the endogenous peptides endomorphin-1 and endomorphin-2 in the Gσ₁-protein pathway, and a partial agonist for β-arrestin recruitment (Supplementary Fig. 5a,b). Further, the pharmacological characterization revealed that although DIPP-NH₂ shows a weak partial agonist activity for both Gσ₁-protein and β-arrestin pathways at the human δ-OR (Supplementary Fig. 5c,d), Schild analysis confirmed its antagonist activity profile with respect to the prototype peptide agonist DADLE, which is structurally related to the endogenous peptide agonist enkephalin (H-Tyr-Gly-Gly-Phe-Met-Leu-OH) (Supplementary Fig. 5e,f).

The δ-OR–DIPP-NH₂ structure also reveals important features of the peptide-recognition site, beyond the naltrexol-defined pocket in previous δ-OR structures. The Phe₃ aromatic side chain of DIPP-NH₂ reaches back toward the receptor core and interacts with the hydrophobic side chain of Leu₁₂⁵, just below ECL2, as well as with the carbon atoms of Tyr₁₂⁹ and Asp₁₈³ side chains (Figs. 1 and 2). Although the Phe₃ side chain is not involved in other hydrophobic interactions, its role in DIPP-NH₂ binding to δ-OR is likely to shield the salt bridge between the N-terminal amine and Asp₁₂⁸ from solvent, thus stabilizing this ionic interaction. Outside of the pocket concealing H-Dmt-Tic-Phe₃, the terminal Phe₄-NH₂ group in its major conformation is found forming two hydrogen bonds to the main chain carbonyl and nitrogen atoms of Leu₂⁰. The side chain of Phe₄ rests against Met₁⁹⁹, which together with Val₁₉⁷ forms a hydrophobic patch on the δ-OR ECL2 β-sheet. The equivalent positions at μ-OR are occupied by charged or polar residues, thus suggesting that the chemical character of residues on ECL2 may be important for OR peptide selectivity (Fig. 2a). Superimposition of μ-OR bound to β-FNA and δ-OR–DIPP-NH₂ structures show a clash between μ-OR Thr₂₁⁸ and DIPP-NH₂, thus rationalizing the shift of ECL2 in the δ-OR–DIPP-NH₂ structure (Fig. 2a,c).

Currently, understanding of the structural determinants for peptide binding to ORs and GPCRs in general is limited because the only other GPCR structure bound to an endogenous peptide is the structure of neuropeptide Y (NPY) receptors (ref. 19). The δ-OR–DIPP-NH₂ structure presented here offers an opportunity to examine the binding mode of a prototype peptide analog and provides a structural platform for the rationalization of structure–activity relationship studies of numerous other reported peptides with distinct pharmacological properties. Because the structural relationship between equivalent positions of functional groups in small-molecule ligands and peptides is often not easy to define, the δ-OR–DIPP-NH₂ structure should prove useful for further understanding of OR function and selectivity. Furthermore, this structure is of the utmost interest for structure-based drug-design efforts, given the potent mixed δ-OR antagonist and μ-OR agonist activity of DIPP-NH₂, a profile known to attenuate opioid side effects. Thus, the structure of the δ-OR–DIPP-NH₂ complex provides a structural basis for development of both peptidic and nonpeptidic ligands as drugs for treatment of pain pathologies through opioid-based therapy.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 4RWA (synchronized structure) and 4RWD (XFEL structure).

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.F. designed, optimized and purified δ-OR receptor constructs for structural studies, crystallized the receptor in LCP, collected and processed XFEL data; C.B. synthesized peptide ligands for structural and signaling studies; P.G. performed signaling studies, analyzed the data and wrote the paper; N.A.Z. collected and processed XFEL data; A.I., H.Z. and W.L. collected XFEL data and helped with sample preparation; K.G. synthesized peptide ligands for structural and signaling studies; O.M.Y. refined the detector geometry and contributed to XFEL data processing; J.C., C.E.C., R.F. and P.F. collected and analyzed XFEL data and helped with biophysical characterization of crystals at LCLS; D.T. and P.W.S. helped with manuscript preparation; B.L.R. supervised the pharmacology studies, analyzed the data and wrote the paper; S. Ballet supervised the peptide synthesis and screening studies, synthesized peptide ligands for structural studies and wrote the paper; V.K. analyzed the data and wrote the paper; R.C.S. determined the overall project strategy; analyzed the data and wrote the paper; V.C. determined the overall project strategy and provided management, supervised XFEL data collection, analyzed the data and wrote the paper with contributions from all other coauthors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Peptide synthesis. The peptide DIPP-NH₂ was synthesized manually via standard solid-phase peptide synthesis with Fmoc-Rink amide AM resin (Iris Biotech) as solid support (0.4 mmol, 625 mg) and Nα-Fmoc- or Nβ-Boc-protected amino acids (Chem-Impx International; Fmoc-Phe-OH purchased from Novabiochem). The coupling reactions were performed with 3 eq. of amino acid, 3 eq. of TBTO (1.2 mmol, 385 mg) and 9 eq. of DIPEA (3.6 mmol, 595 µL) in DMF, for 1.5 h. Fmoc deprotections were realized by means of 20% 4-Me-piperidine in DMF (5 + 15 min). (All solvents were reagent-grade purity.) Boc-Dmt-OM was used as the last amino acid in the sequence in order to recover the fully deprotected peptide after cleavage from the resin. The coupling of this amino acid was performed with 3 eq. of DIC (1.2 mmol, 188 µL) and 3 eq. of HOBt (1.2 mmol, 162 mg) as coupling reagents in order to avoid side reactions that could occur when a TBTU/DIPEA mixture is used. The cleavage of the peptide from the resin and the removal of the Boc protecting group were achieved with a mixture of TFA/TES/H₂O (10:1, 95:2.5:2.5 (v/v) for 3 h). After evaporation of the cleavage mixture, the crude peptide was obtained.

Final purification was performed by reverse-phase (RP) semipreparative HPLC (Gilson; SUPELCO Discovery BIO Wide Pore RP C-18 column, 25 cm × 2.1 mm, 10 µm), and 96 mg of the desired pure compound was isolated (yield, 31%). A purity of more than 98% was determined by analytical RP-HPLC (Agilent 1100 Series system with a SUPELCO Discovery BIO Wide Pore RP C-18 column, 15 cm × 2.1 mm, 3 µm, with UV detection at 215 nm). The structure of the compound was confirmed by electrospray ionization mass-spectrometry (ESI-MS) (Micromass Q-ToF Micro spectrometer).

Peptide characterization of H-Dmt-Tic-Phe-NH₂ was done by HPLC standard gradient: t₂₅ = 13.13 min. ESI-MS [M + H+] m/z = 662.47 (calculated for C₃₅H₄₄N₇O₂₂ 662.33).

Cloning, expression and purification of ß-OR. The WT human ß-OR gene (OPRD1; UniProt P41143) was synthesized by DNA20 with codon optimization for expression in Spodoptera frugiperda (Spf) and then cloned into a modified pFastBac1 vector (Invitrogen) containing an expression cassette with a hemagglutinin signal sequence followed by a Flag tag, a His₁₀ tag and a TEV protease-recognition site at the N terminus. 34 amino acids were deleted from the C terminus (residues 339–372), and 38 residues of the N terminus (residues 1–38) of ß-OR were replaced with the thermostabilized apocytochrome b₅₆₅-RIL from Escherichia coli (M7W, H102I and R106L) (BRIL)26 with splicing by overlap extension PCR. Recombinant baculoviruses were generated with the Bac-to-Bac system (Invitrogen) and were used to infect 5âte insect cells at a density of 2 × 10⁶ cells/ml at a multiplicity of infection of 5. Infected cells were grown at 27 °C for 48 h before being harvested, and the cell pellets were stored at −80 °C.

Receptor was solubilized from isolated membranes in 0.75% (v/v) n-dodecyl-β-D-maltopyranoside (DDM; Anatrace) and 0.15% (w/v) cholesteryl hemisuccinate (CHS; Sigma) and was purified by metal-affinity chromatography as previously described 13.

The protein was then treated overnight with histidine-tagged TEV protease to cleave the N-terminal histidine tag and Flag tag. TEV protease and the cleaved N-terminal fragment were removed by incubation with TALON IMAC resin for 1 h at 4 °C. Purified receptor in 50 mM HEPES, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 0.03% (w/v) DDM, 0.006% (w/v) CHS and 50 mM DIPP-NH₂ was concentrated to 40 mg/ml with a 100-kDa molecular weight—cutoff Vivaspin centrifuge concentrator (GE Healthcare). Protein purity and monodispersity were tested by SDS-PAGE and analytical size-exclusion chromatography (aSEC).

Peptide characterization of H-Dmt-Tic-Phe-NH₂ was done by HPLC standard gradient: t₂₅ = 13.13 min. ESI-MS [M + H+] m/z = 662.47 (calculated for C₃₅H₄₄N₇O₂₂ 662.33).

Cryocrystallization of BRIL-Δ56-OR–DIPP-NH₂ for XFEL data collection. Initial LCP crystallization screening of BRIL-Δ56-OR–DIPP-NH₂ was performed with an NT8-LCP robot (Formulatrix) in 96-well glass sandwich plates as described above to identify conditions that would generate small crystals (5–10 µm) (Supplementary Table 1). Crystals for XFEL data collection were obtained in an incubator/imagery (RockImager 1000, Formulatrix) at 20 °C. Diffraction-quality crystals of an average size of 50 × 30 × 30 µm (Supplementary Fig. 1b) were obtained within ~10-d in 25–28% (v/v) PEG 400, 0.12 to 0.2 M NaCl, 100 mM MES buffer, pH 6.0, 1 mM DIPP-NH₂ and 5% (v/v) of one of the following additives: 30% glycerol, 1.0 M glycine or 0.01 M reduced l-glutathione/0.01 M oxidized l-glutathione. Crystals were harvested directly from LCP with 50 µm MiTeGen micromounts and were immediately flash frozen in liquid nitrogen.

Synchrotron data collection and processing. Crystalllographic data were collected on the 3ID-B beamline (GM/CA CAT) of the Advanced Photon Source at the Argonne National Laboratory with a 10-µm collimated minibeam at a wavelength of 1.033 Å and a MarMosaic 300 detector. To reduce radiation damage, crystals were replaced after collection of 5–10 frames at 2-s exposure and 1.0° oscillation with an unattenuated beam. Data sets from 21 different crystals were integrated, scaled and merged together with HKL2000 (ref. 22) (Supplementary Table 1).

Synchrotron structure determination and refinement. The initial molecular-replacement solution was obtained by Phaser23, with the 1.8-Å ß-OR receptor structure (PDB 4N6H)23 with deleted BRIL fusion, and BRIL from A₂₅-AR (PDB 4E1I)24 as independent search models. The resulting BRILΔ56-OR–DIPP-NH₂ model was refined by manually building in the excessive 2Fᵩ - Fᵩ density, and repetitive cycling between COOT25, REFMAC5 (ref. 26) and simulated annealing with PHENIX 27 until convergence. The final model contains 93.6% residues in favored and 6.4% in allowed Ramachandran-plot regions. The data collection and refinement statistics are shown in Supplementary Table 1.

Cryocrystallization of BRILΔ56-OR–DIPP-NH₂ for XFEL data collection. Initial LCP crystallization screening of BRIL-Δ388-OR–DIPP-NH₂ was performed with an NT8-LCP robot (Formulatrix) in 96-well glass sandwich plates as described above to identify conditions that would generate small crystals (~5 µm) (Supplementary Fig. 1 cd). Crystals for XFEL data collection were obtained in Hamilton gas-tight syringes with the following procedure 28. Purified BRILΔ56-OR–DIPP-NH₂ complex at a concentration of 40 mg/mL was reconstituted in LCP as described above. Approximately 5 µL of protein-laden LCP was carefully injected as a continuous filament of ~400 µm in diameter into a 100-µL syringe filled with 60 µL of precipitant solution: 0.1 M MES, pH 6.0, 50–180 mLi/m lithium citrate and 30–32% (v/v) PEG 400, and incubated for 24 h at 20 °C (Supplementary Fig. 1f). After crystals had formed, excess precipitant solution was carefully removed, and this was followed by the addition of ~3 µL of 0.7 M Ag to absorb the residual precipitant solution. The crystal samples were characterized at LCLS by optical microscopy (Supplementary Fig. 1e).

XFEL data collection and processing LCP-SEX data collection was performed with the CXI instrument at the Linac Coherent Light Source (LCLS) at SLAC National Accelerator Laboratory. The LCLS was operated at a wavelength of 1.56 Å (7.95 keV), delivering individual X-ray pulses of 35–fs duration and 10¹² photons per pulse, focused into a spot size of approximately 1.5 µm in diameter with a pair of Kirkpatrick–Baez mirrors. Protein microcrystals in LCP medium were injected at room temperature inside a vacuum chamber into the beam focus region with the LCP injector with a 50-µm-diameter nozzle at a flow rate of 0.17 µL/min. Microcrystals ranged in size from 1 to 10 µm, with an average size of 5 × 2 × 2 µm² (Supplementary Fig. 1e). Single-shot diffraction patterns of randomly oriented crystals (Supplementary Fig. 3) were recorded at 120 Hz, with the 2.3-megapixel Cornell-SLAC Pixel Array Detector (CSPAD). The sample-to-detector distance of ~100 mm corresponded to a maximum resolution of 1.8 Å (at the corner of the detector), and the beam was attenuated to 9% (9 × 10¹¹ ph/pulse) of full intensity to avoid detector saturation, corresponding to a radiation dose of up to ~46 MGy deposited in each crystal.

A total of 1,967,359 detector readouts were collected, of which 125,458 were identified as potential single-crystal hits with more than 15 potential Bragg peaks with Cheetah29, corresponding to an average hit rate of 5.9%. Autoindexing and structure-factor integration of the crystal hits were performed with CrysFEL30 (version 0.5.4). Peak-detection parameters were extensively optimized for Cheetah, and experimental geometry was refined with CrysFEL. The overall time of data collection from seven samples with a total volume of 50 µL was about
4.6 h and yielded 36,083 indexed patterns. Data-resolution cutoff was selected on the basis of the behavior of the Pearson correlation coefficient (CC1/2 = 0.538 in the highest-resolution shell).

**XFEL structure determination and refinement.** The XFEL structure was determined with the same procedure used to determine the synchrotron structure described above. The final model contains 98.5% residues in favored and 1.5% in allowed Ramachandran-plot regions. The data collection and refinement statistics are shown in [Supplementary Table 1](#).

**δ-OR β-arrestin–recruitment Tango assay.** Arrestin recruitment assays were performed as previously described with a modified version of the original Tango assay.

**µ-OR β-arrestin–recruitment bioluminescence resonance energy transfer assay.** µ-OR β-arrestin–translocation assay was performed with a bioluminescence resonance energy transfer (BRET)-based assay as originally described. In brief, HEK293T cells (ATCC CRL-11268) were cotransfected with cDNA encoding the µ-OR fused at the C terminus with the Renilla reniformis luciferase (Luc8) and with the Venus-tagged β-arrestin-2 and G protein–coupled receptor kinase 2. 24 h post-transfection, cells were distributed on clear-bottom white poly-lysine–coated 96-well plates. The next day, cells were rinsed once with PBS and incubated 10 min in 90 µl of assay buffer (1 × HBSS and 20 mM HEPES, pH 7.40) containing 5 µM coelenterazine-h. Then cells were stimulated for 15 min by the addition of 10 µl of 10× drugs diluted in assay buffer, and emitted light was collected with a Mithras LB-940 reader (Berthold Technologies).

**cAMP assays.** cAMP was quantified with a luciferase-based Glosensor bioassay (Promega) as previously described in transiently transfected HEK293T cells. Data were normalized compared to the agonist DAMGO for the µ-OR and DADLE for the δ-OR, and a nonlinear regression was applied with the sigmoidal dose-response function.

**DIPP-NH$_2$ binding assays.** Binding of DIPP-NH$_2$ to δ-OR and µ-OR was performed as previously described.

**Statistical analysis.** Data for ligand binding and signaling assays are presented as mean ± s.e.m. from three independent experiments (n = 3) conducted in quadruplicate. Data were analyzed with GraphPad Prism.

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