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Discovery of a Novel Selective Kappa-Opioid Receptor Agonist Using Crystal Structure-Based Virtual Screening

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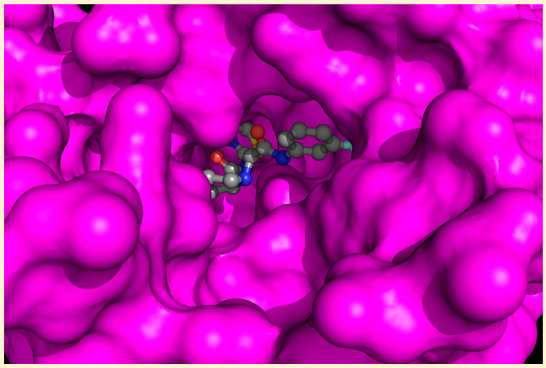
Supporting Information



ABSTRACT: Kappa-opioid (KOP) receptor agonists exhibit analgesic effects without activating reward pathways. In the search for nonaddictive opioid therapeutics and novel chemical tools to study physiological functions regulated by the KOP receptor, we screened in silico its recently released inactive crystal structure. A selective novel KOP receptor agonist emerged as a notable result and is proposed as a new chemotype for the study of the KOP receptor in the etiology of drug addiction, depression, and/or pain.

INTRODUCTION

Opioids remain the most widely prescribed and abused class of medicines.[1,2] Addiction is not the only limiting factor for the effective use of these compounds as powerful painkillers, antitussives, antidepressants, or antipruritic agents. In addition to social and legal issues associated with their use for nonmedical, recreational purposes, several adverse effects (e.g., dysphoria, constipation, respiratory depression, nausea, vomiting, etc.)[3] hinder their clinical usefulness and justify the enormous effort put forth by numerous investigators over the years to discover safer opioid therapeutics and/or nonaddictive medications. Notwithstanding the continued development of many compounds with opioid activity, ranging from useful agents in the clinic to important chemical tools to study the endogenous opioid system, a safe, nonaddictive, and effective opioid drug is yet to be discovered.[3]

Notable members of the superfamily of G protein-coupled receptors (GPCRs), mu-, delta-, and kappa-opioid (or MOP, DOP, and KOP) receptor subtypes[4−7] are natural targets for the majority of opioid ligands. The most clinically used opioid drugs act as agonists at the MOP receptor[3] and exert addiction liability through activity at this receptor.[8,9] Thus, it has been proposed that high-affinity selective ligands of the DOP and KOP receptors would provide more effective routes to discovering nonaddictive analgesics.[10,11] In particular, KOP receptor agonists have been shown to be unable to activate the reward pathway[12] while still acting as effective pain suppressors on the central nervous system (CNS) and/or the periphery,[11] most likely through the $G_{i/o}$ protein-mediated inhibition of cAMP production,[13] the blockade of calcium channels,[14] and/or the activation of the inward rectifier potassium channels.[15] Unfortunately, the KOP receptor agonists developed to date are not ideal drugs as they exert side effects such as dysphoria.[16] However, KOP receptor-mediated dysphoric effects have recently been attributed to the activation of the p38 MAPK pathway following arrestin recruitment to the activated KOP receptor.[16−18] Therefore, KOP receptor-selective G-protein biased agonists, which do not recruit arrestin, have been proposed to be more effective analgesics, without the adverse effects triggered by the arrestin pathway.[18] We have recently reported on such a functionally selective G protein-biased KOP receptor ligand:[19] 6′-guanidinonaltrindole (6′-GNTI). Although this morphine-derivative ligand is a promising lead compound for nonaddictive analgesics acting at the KOP receptor with reduced liability for dysphoria, its effective use as a drug is severely limited by its physicochemical properties and its inability to cross the blood−brain barrier.

The lack of a detailed molecular-level understanding of the interactions between opioids and their receptors has hindered successful receptor-based drug design. By revealing how opioid ligands bind to their receptors, recent high-resolution crystal structures of all four opioid receptor subtypes, i.e., the MOP,[20] DOP,[21] KOP,[22] and nociceptin/orphanin FQ[23] receptors, offer

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an unprecedented opportunity to discover novel chemotypes targeting these proteins that might eventually be developed into more efficacious therapeutics.[24,25]

In the search for these nonaddictive therapeutics targeting opioid-receptors, we screened in silico over 4.5 million commercially available, "lead-like" small molecules accessible in ready-to-dock three-dimensional format in the ZINC database,[26] based on complementarity with the crystallographic binding mode of JDTic into the KOP receptor crystal structure.[22] To the best of our knowledge, this is the first virtual screening study at the KOP receptor using its recently released crystal structure.[22] The study led to the identification of 4 novel small-molecule chemotypes, out of 22 tested molecules, acting at the KOP receptor. The *S*-stereoisomer of one of these compounds was further characterized as a novel selective KOP receptor ligand with agonistic activity at this receptor, and as such, it represents a promising candidate for structure-based drug design.

METHODS

Small-Molecule Subset, Docking, Selection, and Novelty. We used the lead-like subset version of the ZINC database[26] that was accessible online on February 2, 2012, when the molecular docking study was performed. This subset version contained ∼4.5 million commercially available small-molecules selected using the filtering criteria specified on the ZINC database Web site. Molecular docking at chain A of the recently released inactive KOP receptor structure (PDB ID: 4DJH[22]), following removal of all nonprotein atoms, was performed with DOCK3.6.[27−30] The atom positions of the JDTic crystallographic ligand within the KOP receptor binding pocket were replaced by 45 spheres that had been labeled for chemical matching based on the local protein environment. Default parameters, i.e., a bin size of 0.2 Å, a bin size overlap of 0.1 Å, and a distance tolerance of 1.2 Å for both the binding site matching spheres and each docked small-molecule from the lead-like subset, were used for ligand conformational sampling. Partial charges from the united atom AMBER force field[31] were used for all receptor residues with the exception of Asp138 in transmembrane helix 3. The dipole moment of this residue was increased by 0.4 per polar atom to favor identification of small molecules that would form ionic interactions with this residue.[32] The KOP receptor was kept rigid while each small molecule was docked into the binding pocket in an average of 3073 orientations relative to the receptor and an average of 2132 conformations for each orientation. A score corresponding to the sum of the receptor−ligand electrostatic and van der Waals interaction energies, corrected for ligand desolvation, was assigned to each docked molecule and configuration within the KOP receptor binding pocket. The specific energy estimates were obtained as we recently described for an analogous study.[33] The best scoring conformation of each docked molecule was further subjected to 100 steps of energy minimization with the protein residues kept rigid. Twenty-two compounds, termed here MCKK-1−22, and listed in Supporting Information Table S1, were selected from visual inspection of the 500 top-scoring docked compounds (Supporting Information Table S2) based on criteria discussed in Results and Discussion. Similarity between these molecules and the 9934 opioid receptor ligands that are annotated in the ChEMBL database [https://www.ebi.ac.uk/chembl/] (Supporting Information Table S1) was quantified using an in-house script in *R* language that calculates Tanimoto coefficients (T_c) to the nearest neighbors based on extended connectivity fingerprint maximum distances 4 (ECFP4) and the protocol we recently reported.[33] T_c values range from 0 to 1, with the 0 value indicating maximally dissimilar compounds and 1 indicating maximally similar ones.[34] As suggested in the literature,[35] molecules are considered reasonably similar if their T_c value is above 0.40. Molecules for testing were purchased from commercial vendors. Specifically, compounds MCKK-1, MCKK-4, MCKK-8, MCKK-15, MCKK-18, and MCKK-21−22 were obtained from ChemBridge, MCKK-2 from the National Cancer Institute of the National Institutes of Health, MCKK-3 from Labotest, MCKK-5−7, MCKK-9−13, MCKK-16−17, and MCKK-19 from Enamine, MCKK-14 from Florida Heterocyclic Compounds, and MCKK-20 from Molecular Diversity Preservation International.

Constructs for Expression Vectors and Transfection. The cDNAs for human KOP (hKOP) receptor and the G protein $G\alpha_{oB}$ were obtained from the Missouri S&T cDNA Resource Center. For arrestin recruitment experiments, full-length *Renilla* luciferase 8 (RLuc8, provided by S. Gambhir) was fused in-frame to the C terminus of the hKOP receptor in the pcDNA3.1 vector. The following human G protein constructs were provided by Gales:[36,37] $G\alpha_{oB}$ with RLuc8 inserted at position 91 ($G\alpha_{oB}$-RLuc8); untagged $G\beta_1$ ($\beta1$); untagged $G\gamma_2$ ($\gamma2$). The human $\gamma2$ subunit was fused to full-length mVenus at its N terminus (mVenus-$\gamma2$), and we used the fusion construct human arrestin3−mVenus previously described.[38] All constructs were confirmed by sequencing analysis. A total of 20 μg of plasmid cDNA (e.g., 0.2 μg of hKOR-RLuc8, 15 μg of arrestin3-mVenus, and 4.8 μg of pcDNA3.1) was transfected into HEK-293T cells using polyethylenimine (Polysciences Inc.) in a 1:3 ratio in 10-cm dishes. Cells were maintained in culture with DMEM supplemented with 10% FBS. The transfected ratio among receptor, Gα, $\beta1$, and $\gamma2$, or arrestin was optimized by testing various ratios of plasmids encoding the different sensors. Experiments were performed 48 h after transfection.

Membrane Preparations and Binding Assays. Two days after transfection with human KOP receptor and $G\alpha_{oB}$, HEK293T cells were lysed and membranes were prepared in HEPES buffer (NaCl 140 mM, KCl 5.4 mM, HEPES 25 mM, EDTA 1 mM, $MgCl_2$ 2 mM, BSA 0.006%, pH 7.4) using a Polytron homogenizer. Membranes were incubated with 3*H*-diprenorphine (0.3 nM) (PerkinElmer) at room temperature for 1 h in a final volume of 1 mL, in the absence or presence of various concentrations of each small-molecule selected from the virtual screening. Membranes were then harvested using a Brandel cell harvester through a Whatman FPD-24 934AH glass-fiber filter and washed three times with ice-cold wash Buffer (Tris-HCl 10 mM, NaCl 120 mM, pH 7.4). Nonspecific binding was determined using 400 nM of NorBNI.

BRET-Based G Protein Activation, Arrestin Recruitment, and cAMP Accumulation Assays. BRET was performed as described.[39] Briefly, two days after transfection, cells were harvested, washed, and resuspended in a phosphate-buffered saline (PBS) solution. Approximately 200 000 cells/well were distributed in 96-well plates, and 5 μM coelenterazine H (luciferase substrate) was added to each well. Five minutes after the addition of coelenterazine H, ligands were added to each well. After 2 min for G protein activation or 5 min for arrestin recruitment, the BRET signal was determined by quantifying and calculating the ratio of the light emitted by mVenus, the energy acceptor (510−540 nm), over that emitted

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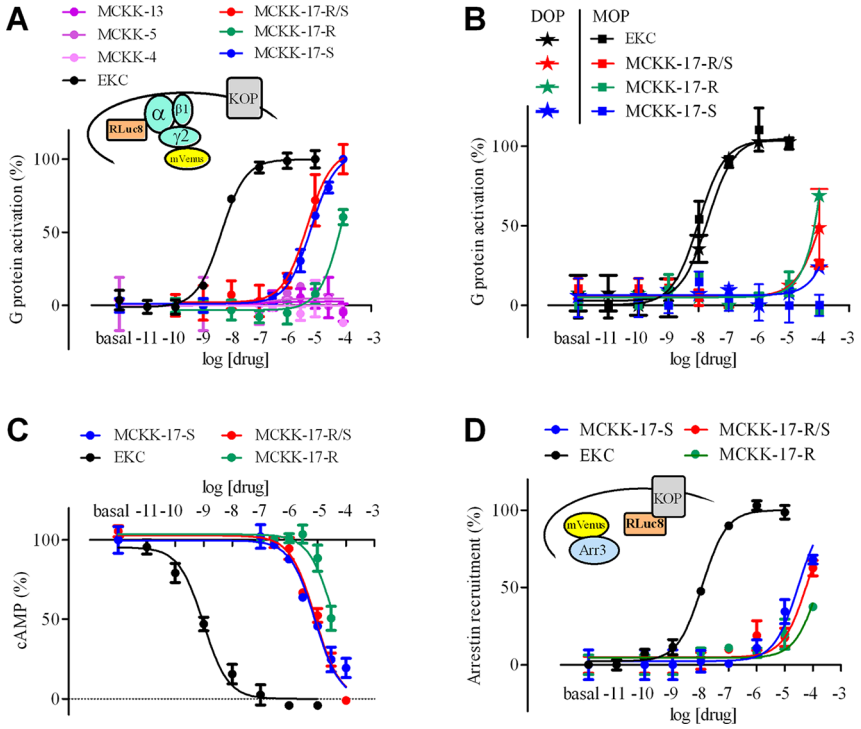

Figure 1. MCKK-17-*S* is a selective hKOP receptor agonist. (A and B) hKOP receptor (A), DOP or MOP receptors (B) was coexpressed with $G\alpha_{oB}$–RLuc8, β1, and mVenus–γ2 to assay G protein activation. (A) Only MCKK-17-*R*/*S* exhibited agonistic activity at the hKOP receptor, and MCKK-17*S* was the more active stereoisomer. (B) MCKK-17*S* is selective for hKOP relative to MOP and DOP receptors. (C) hKOP receptor was coexpressed with a BRET-based CAMYEL sensor to assay inhibition of forskolin-stimulated cAMP accumulation. (D) hKOP receptor, fused to RLuc8, was coexpressed with arrestin3 (*Arr3*) fused to mVenus to assay arrestin recruitment to the activated receptor. Error bars indicate SE.

by RLuc8, the energy donor (485 nm). The drug-induced BRET signal was normalized, taking the E_{max} of the ethylketocyclazocine (EKC)-induced response as 100%. To measure cAMP accumulation, we used a BRET-based cAMP in a previously described YFP-Epac-RLuc (CAMYEL) assay.[40] $G\alpha_{oB}$, β1, and γ2 were coexpressed to enhance the signal-to-noise ratio, and the cells were treated for 5 min with 100 μM forskolin prior to stimulation.[40] The data were normalized and represented as the percentage of forskolin-stimulated cAMP accumulation with 0 defined as the maximal inhibition elicited by EKC.

Chemical Synthesis of MCKK-17 Stereoisomers. All reagents purchased from chemical suppliers were used without further purification and reactions monitored using thin-layer chromatography (TLC) on 0.25 mm Analtech GHLF silica gel plates using EtOAc/*n*-hexanes and visualized at 254 nm. Column chromatography was performed on silica gel (40–63 μm particle size, 230–400 mesh) from Sorbent Technologies (Atlanta, GA). NMR spectra were recorded on either a Bruker DRX-400 with a H/C/P/F QNP gradient probe or a Bruker Avance AV-III 500 with a dual carbon/proton cryoprobe using δ values in parts per million as standardized from tetramethylsilane (TMS) and J (Hz) assignments for ^{1}H resonance coupling and ^{13}C fluorine coupling. High resolution mass spectrometry data were collected on a LCT Premier (Waters Corp.) time-of-flight mass spectrometer. Analytical HPLC was performed on an Agilent 1100 Series Capillary HPLC system with diode array detection at 254.8 nm on a CRIRALCEL OD-H column (4.6 × 150 mm), Daicel Chemical Industries, Ltd., using isocratic elution in 97% hexanes and 3% 2-propanol at a flow rate of 1.25 mL/min. General procedures for the synthesis of *tert*-butyl 2-(thiazol-2-ylcarbamoyl)-pyrrolidine-1-carboxylate (2*S* and 2*R*), as well as 1-(2-(3-fluorophenylamino)-2-oxoethyl)-*N*-(thiazol-2-yl)pyrrolidine-2-carboxamide (MCKK-17S and MCKK-17R) are provided in the Supporting Information.

RESULTS AND DISCUSSION

Structure-Based Identification of Novel Chemotypes Targeting the KOP Receptor. We screened in silico 4,554,059 commercially available, lead-like compounds from the ZINC database[26] based on complementarity with the crystallographic binding mode of JDTic into the KOP receptor binding pocket. The 500 top-scoring docking hits (Supporting Information Table S2; 0.01% of the docked library) were visually inspected and prioritized based on features that an automatic molecular docking screen does not take into account. Specifically, molecules were selected based on the following criteria: (a) chemotype diversity; (b) the presence of polar interactions between the ligand and the Asp138 residue; (c) interactions with KOP receptor residues in the binding pocket that are different in DOP and MOP receptors; (d) limited flexibility; (e) different binding modes from classical alkaloids as revealed by DOP[21] and MOP[20] receptor crystal structures; and (f) purchasability, i.e., molecules were readily available for purchase. On the basis of these criteria, 22 small molecules were purchased from the set of 500 highest-scored compounds. These molecules, labeled MCKK-1–22 in Supporting Information Table S1, corresponded to the DOCK scoring ranks 1, 80, 87, 97, 111, 127, 137, 210, 253, 269, 276, 346, 347, 360, 379, 402, 403, 404, 411, 427, 452, and 472, respectively. As shown in Table S1, these compounds were found to be significantly different from annotated opioid receptor ligands in the ChEMBL database, as indicated by small ECFP4-based T_c

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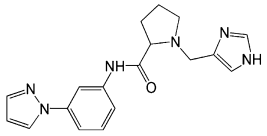
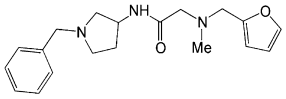
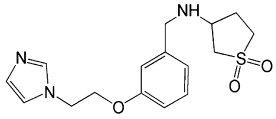
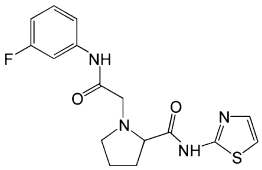
Table 1. Active Compounds and Their Corresponding Chemical Structure, DOCK Scoring Rank from the Virtual Screening Experiment, and Binding Affinity Values (K_i)[a]

Compound (Rank)	Structure	Ki (μM) KOP	DOP	MOP
MCKK-4 (97)		290 ± 210	640 ± 190	910 ± 840
MCKK-5 (111)		130 ± 81	180 ± 190	430 ± 140
MCKK-13 (347)		450 ± 27	300 ± 99	>10000
MCKK-17 (403)		17R: 2900 ± 960 17S: 120 ± 38	17R: 290 ± 320 17S: >10000	17R: 510 ± 640 17S: >10000

[a]Values are the means ± S.E.M. (n = 3).

values.[35] These data confirm the chemotype novelty of all selected agents.

One of the Top-Scoring Docked Molecules: Selective Agonist at the KOP Receptor. The primary experimental testing of MCKK-1−22 consisted of performing a competitive inhibition binding assay at the hKOP receptor. Membranes of HEK293T cells transfected with the hKOP receptor and $G\alpha_{oB}$ were prepared and incubated with 3*H*-diprenorphine (0.3 nM) in the absence or presence of 10 or 100 μM of each small-molecule from the virtual screening. Four molecules, MCKK-4, MCKK-5, MCKK-13, and MCKK-17 partially but significantly inhibited 3*H*-diprenorphine binding (Supporting Information Figure S1) at 100 μM, and their properties were therefore further investigated.

To assess whether any of these molecules had agonistic activity, we used a BRET-based G protein activation assay where the hKOP receptor was coexpressed in HEK293T cells with $G\alpha_{oB}$−RLuc8, β1, and mVenus−γ2, as discussed in Methods. The drug-induced BRET signal is interpreted as a dissociation of and/or conformational change within the $G\alpha\beta\gamma$ complex, and thus, as the activation of the coexpressed G protein. Among the selected molecules, only the racemic mixture MCKK-17R/S activated $G\alpha_{oB}$ with a potency of 8.3 ± 4.0 μM (Figure 1A). Thus, we proceeded to the chemical synthesis of the *R*- and *S*-stereoisomers of MCKK-17 using commercially available *N*-(*tert*-butoxycarbonyl)-L-proline (**1*R***) or *N*-(*tert*-butoxycarbonyl)-D-proline (**1*S***) (Supporting Information Figure S2) to identify the active molecule. The appropriate proline was coupled to 2-aminothiazole using 1,1′-carbonyldiimidazole (CDI) in CH_2Cl_2 under anhydrous conditions[41,42] to afford the corresponding Boc-protected thiazoles (**2*R*** and **2*S***). Removal of the Boc group under acidic conditions followed by alkylation with 2-chloro-*N*-(3-fluorophenyl)acetamide[43,44] under basic conditions in DMF overnight at 80 °C gave stereoisomers MCKK-17*S* and MCKK-17*R*. The purity of MCKK-17*S* and MCKK-17*R* was determined to be at least 99% by integration of the UV trace from chiral HPLC (data not shown). MCKK-17*S* resulted in the most active stereoisomer at the hKOP receptor. Indeed, MCKK-17*S* displayed full agonism relative to EKC at the hKOP receptor with an EC_{50} of 7.2 ± 3.8 μM, whereas MCKK-17*R* displayed a potency of only 120 ± 9 μM. None of the other molecules, MCKK-4, MCKK-5, and MCKK-13, significantly activated G_{oB} (Figure 1A), suggesting that, in contrast to MCKK-17*R*/*S*, those molecules are antagonists at the hKOP receptor.

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To assess the degree of selectivity of MCKK-4, MCKK-5, MCKK-13, MCKK-17*R*, or MCKK-17*S* for the hKOP over the DOP and MOP receptors, we performed competitive inhibition of 3*H*-diprenorphine (0.3 nM) binding in the absence or presence of various concentrations of each molecule and determined their K_i values for each receptor (Table 1). EKC was used as a reference at all three receptors with recorded K_i values of 20 ± 6 nM at hKOP receptor, 250 ± 31 nM at the DOP receptor, and 170 ± 100 nM at the MOP receptor. Consistent with the first experimental testing (Supporting Information Figure S1), the aforementioned five molecules displayed a relatively weak affinity (K_i values) at the hKOP receptor, between 100 and 500 μM (Table 1). Among them, only MCKK-17S exhibited selectivity for the hKOP receptor with a measured affinity of 120 ± 38 μM at this receptor, and no detectable affinity at DOP and MOP receptors (>1000 μM).

To further assess the selectivity of MCKK-17*S* for the hKOP receptor (Table 1), we also investigated whether the racemic mixture MCKK-17*R*/*S*, as well as the two stereoisomers, displayed agonistic activity at the DOP and MOP receptors (Figure 1B). Neither the racemic nor the MCKK-17 stereoisomers displayed significant activity at the MOP receptor, and they only weakly activated the DOP receptor (>1000 μM). Notably, MCKK-17*R*, the less active steroisomer at the hKOP receptor, was the most active enantiomer at the DOP receptor. In contrast, MCKK-17*S* did not significantly activate the DOP receptor, confirming its selectivity at the hKOP receptor. These results were confirmed using a BRET-based cAMP accumulation inhibition assay (Figure 1C) to monitor the agonistic activity of the selected molecules at the hKOP receptor (Figure 1C), as well as at the DOP and MOP receptors (Supporting Information Figure S3).

Finally, we investigated whether MCKK-17 and the corresponding stereoisomers could recruit arrestin. MCKK-17*R*/*S* and MCKK-17*S* recruited arrestin3, with potencies of 160 ± 38 and 120 ± 47 μM, respectively, whereas MCKK-17*R* only weakly recruited arrestin3 at the highest concentration (potency >1000 μM) (Figure 1D), consistent with its weaker potency at the hKOP receptor for G protein activation.

CONCLUSIONS

Although several nonpeptidic selective molecules targeting the KOP receptor have been developed, we are still far from a therapeutically effective KOP receptor drug. Our structure-based virtual screening and compound selection criteria yielded the discovery of a novel small-molecule chemotype that acts as a selective, full agonist at the KOP receptor. To the best of our knowledge, this is the first time that a virtual screening based on an antagonist-bound GPCR crystal structure has identified an agonist. We were pleased to note that the chemical scaffold of the identified hit refers to a selective KOP receptor compound that has very little similarity with all opioid receptor agonists or antagonists annotated in ChEMBL, and that it has never been reported to be an opioid receptor ligand. In summary, MCKK-17*S* is a promising new lead compound for structure-based ligand optimization aimed at discovering potent nonaddictive analgesics. Although the parent compound MCKK-17*S* is not biased toward G protein activation over arrestin, the chemical scaffold is well suited to structure-guided modifications, raising the prospects of maintaining selectivity while increasing potency and building G protein bias.

ASSOCIATED CONTENT

Supporting Information

General procedures for the synthesis of MCKK-17*S* and MCKK-17*R*. Table S1: List of the 22 tested compounds. Table S2: Details of the 500 top-scoring docked compounds from virtual screening at the KOP receptor. Figure S1: Plot of competitive inhibition of 3*H*-diprenorphine binding at the KOP receptor. Figure S2 Synthetic scheme used to obtain MCKK-17*R* and MCKK-17*S* stereoisomers. Figure S3 shows the cAMP accumulation inhibition curves at DOP and MOP receptors. This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖A.N. and M.-L.R. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): Drs. Filizola, Javitch, and Prisinzano are in the process of filing a provisional patent application through their respective technology transfer offices.

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