Probing ligand recognition of the opioid pan antagonist AT-076 at nociceptin, kappa, mu, and delta opioid receptors through structure-activity relationships

V. Blair Journigan1,2, Willma E. Polgar1, Edward W. Tuan1, James Lu1, Pankaj R. Daga2 & Nurulain T. Zaveri1

Very few opioid ligands binding to the three classic opioid receptor subtypes, mu, kappa and delta, have high affinity at the fourth opioid receptor, the nociceptin/orphanin FQ receptor (NOP). We recently reported the discovery of AT-076 (1), (R)-7-hydroxy-N-((S)-1-(4-(3-hydroxyphenyl)piperidin-1-yl)-3-methylbutan-2-yl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide, a pan antagonist with nanomolar affinity for all four subtypes. Since AT-076 binds with high affinity at all four subtypes, we conducted a structure-activity relationship (SAR) study to probe ligand recognition features important for pan opioid receptor activity, using chemical modifications of key pharmacophoric groups. SAR analysis of the resulting analogs suggests that for the NOP receptor, the entire AT-076 scaffold is crucial for high binding affinity, but the binding mode is likely different from that of NOP antagonists C-24 and SB-612111 bound in the NOP crystal structure. On the other hand, modifications of the 3-hydroxyphenyl pharmacophore, but not the 7-hydroxy Tic pharmacophore, are better tolerated at kappa and mu receptors and yield very high affinity multifunctional (e.g. 12) or highly selective (e.g. 16) kappa ligands. With the availability of the opioid receptor crystal structures, our SAR analysis of the common chemotype of AT-076 suggests rational approaches to modulate binding selectivity, enabling the design of multifunctional or selective opioid ligands from such scaffolds.
residues in the ligand-binding pocket, viz. the address domains of the opioid receptors\textsuperscript{10}. These binding models were further confirmed by site-directed mutagenesis studies\textsuperscript{11,12}, and, together with the SAR and docking studies, provided a sound understanding of the structural and molecular basis of ligand recognition at the opioid receptors, even before the ligand-bound opioid receptor crystal structures were elucidated. Notably, the DOP crystal structure bound to antagonist naltrindole\textsuperscript{13} and the MOP crystal structure bound to antagonist β-FNA\textsuperscript{14}, show that the binding orientation of these antagonists are consistent with binding models previously proposed based on the opioid homology models\textsuperscript{10,12}. The discoveries of highly selective opioid tool ligands from common opioid chemotypes like the morphinans underscore the importance of SAR and receptor structure-based rational chemical modifications to the field of opioid ligand drug design.

We recently reported an opioid antagonist AT-076 (1), which has nanomolar affinity for all four opioid receptor subtypes\textsuperscript{15}. This opioid pan-antagonist is a non-morphinan opioid ligand, containing a phenylpiperidine scaffold and is a close analog of the kappa-selective antagonist JDTic (Fig. 1). The phenylpiperidine moiety in 1 and the (3 R,4 R)-dimethyl-4-(3-hydroxyphenyl)piperidine scaffold in JDTic are common nonmorphinan opioid antagonist pharmacophores, present in other opioid antagonists such as the mu opioid-selective antagonist alvimopan, (Fig. 1) and the NOP antagonists C-24 and SB-612111 (Fig. 1).

The nanomolar binding affinity of AT-076 to all four opioid receptors suggests that AT-076 possesses a chemotype that can bind with high affinity at all opioid receptors and can function as a universal opioid scaffold. We therefore conducted a SAR study to probe the chemical features of AT-076 that play a role in ligand recognition at the four opioid receptors.

AT-076, being a phenylpiperidine-based non-morphinan opioid antagonist, is a close structural analog of the nonmorphinan kappa antagonist JDTic and similar to the phenylpiperidine-based NOP antagonists C-24 and SB-612111 (Fig. 1). Previously, we reported docking models of AT-076 in the KOP and NOP crystal structures (PDB No: 4DJH\textsuperscript{17} and PDB No: 4EA3\textsuperscript{18} respectively), which provided putative binding orientations of AT-076 in the NOP and KOP receptors\textsuperscript{15}. The highest-scoring docked orientation of AT-076 in the NOP binding pocket was similar to the binding orientations of crystallized NOP antagonists C-24 and SB-612111 in the NOP receptor (shown in Fig. 2), such that the aromatic moiety at the 4-position of the piperidine ring (benzofuran ring in C-24, 2,6-dichlorophenyl in SB-612111, and 3-hydroxyphenyl in AT-076) was oriented towards the intracellular end of the binding pocket, consisting of hydrophobic residues Met134\textsuperscript{3,38}, Phe135\textsuperscript{3,37}, Ile219\textsuperscript{3,42} and Val283\textsuperscript{3,55}.

A related aim of our study was to confirm these putative docking orientations through an SAR analysis of AT-076 by introducing rational chemical modifications based on the putative docking poses of AT-076 at the NOP and KOP receptors. Figure 3 shows the structures of analogs 2–16 designed to provide information on the ligand recognition features of AT-076 important for providing high affinity at the four opioid receptors.

We previously reported that the 7-hydroxy group on the tetrahydroisoquinolinyl (Tic) moiety of AT-076 was important for maintaining high binding affinity at all four opioid receptors, because removal of this hydroxy group significantly decreased affinity at all four opioid receptors\textsuperscript{15}. The 3-hydroxy group on the 4-phenylpiperidine moiety however, could be removed with a small loss in binding affinity at the NOP, MOP and KOP, but caused a loss in affinity at DOP receptors. We focused our SAR modifications on the 7-hydroxy and 3-hydroxy groups of AT-076.
to obtain more information on the binding orientations of AT-076 in the opioid receptors, given the known differences in amino acid residues in the transmembrane domains and binding pockets of the four opioid receptors.

Both the NOP and KOP receptors (but not DOP and MOP) contain anionic amino acid residues in their extracellular loop (EL) 2 (between transmembrane helices (TM) 4 and 5), which have been shown to function as the address domains for these receptors and interact with the cationic core residues of their respective endogenous peptide ligands nociceptin and dynorphin. For kappa antagonists such as norBNI and 5′-GNTI, the selectivity-enhancing 'address' interaction occurs with the nonconserved residue Glu297 at the extracellular end of TM6. To explore and confirm binding orientations of AT-076, we introduced various positively charged groups in the 7-hydroxy-Tic moiety by replacing the 7-hydroxy group with amine, guanidine and N-methyl sulfonamide groups; as well as substitution of the Tic-OH heterocycle with lysine and arginine groups. Additionally, for these compounds, the effect of removal of the 7-OH of the Tic moiety was also explored.

As shown in the 2D diagram in Fig. 2, derived from the C-24 and SB-612111-bound NOP receptor crystal structures, the benzofuran and 2,6-dichlorophenyl moieties at the 4-position of the piperidine are oriented towards the intracellular end of the TM binding pocket, surrounded by hydrophobic residues Met134, Phe135, Ile219 and Val283. Docking of AT-076 in the NOP crystal structure also resulted in a similar orientation of the piperidine ring, where the 3-hydroxyphenyl ring at the 4-position of the piperidine ring was
were synthesized as shown in Fig. 6. For the cyano analog 4 deprotection of intermediates afforded 8 and I-8, BOP-mediated coupling of amine I-6, afforded final products 11–13, Boc-deprotection afforded arginine analogs 12 and 13.

**Chemistry**

Cyano (2), amine (3), guanidine (4) and N-methyl sulfonamide (5) substitutions of the 7-hydroxy Tic-OH were prepared as shown in Fig. 4. Selective methylation of (3 R)-2-(tert-butoxycarbonyl)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid I-1 was achieved in quantitative yield using LiOH and dimethyl sulfate, followed by treatment of the free phenol with N-phenyl-bis(trifluoromethanesulfonimide) (PhNTf₂) to trflate I-2. Subsequent Pd-catalyzed cyanation and LiOH deprotection afforded I-3. BOP-mediated amidation of carboxylic acid I-4 with amine (S)-3-(1-(2-amino-3-methylbutyl)piperidin-4-yl)phenol I-5 (prepared according to literature methods) gave I-5. HCl deprotection then afforded 2. Hydrogenation of the nitrile of I-5 with Raney Ni yielded the amine I-6, which upon removal of the Boc group with HCl afforded 3. Amine I-6 was subjected to HgCl₂-mediated guanidation using 1,3-bis (tert-butoxycarbonyl)-2-methyl-2-thiopeudourea to give intermediate I-7, and mono-sulfonated with methyisulfonfyl chloride/pyridine to give intermediate I-8. HCl deprotection of intermediates I-7 and I-8 afforded 4 and 5, respectively.

Analogs bearing positively charged replacements of the Tic-OH, as well as corresponding analogs lacking the 7-hydroxy substitution at the Tic moiety, were synthesized as shown in Fig. 5. For the cyano analog 8, amine I-4 was first coupled with TBDMS-protected carboxylic acid III-1a using standard BOP-mediated coupling to amide III-2a. Corresponding analog III-1b, lacking the 7-hydroxyl at the Tic moiety, was prepared as previously reported. Conversion of the 3-hydroxyl on the phenylpiperidine of III-2a and III-2b to trilates III-3a and III-3b using N-phenyl-bis(trifluoromethanesulfonimide) (PhNTf₂), followed by Pd-catalyzed cyanation furnished III-4a and III-4b in good yields. TBDMS- and Boc-deprotection of intermediates III-4a afforded cyano analog 8 in reasonable overall yield. Reduction of 8 with Raney Ni/H₂(g) then afforded 10. Boc deprotection of III-4b with trifluoroacetic acid yielded cyano analog 9. To obtain analogs 11–13, protected cyano intermediates III-4a and III-4b were reduced to their correspondents 11–13.

**Figure 4.** Synthesis of 2–5. Reagents and conditions: (a) LiOH, Me₂SO₄, acetone, reflux, quantitative; (b) N-phenyl-bis(trifluoromethanesulfonimide), K₂CO₃, THF, 22 h, rt, 50%; (c) ZnCN₂, Pd(PPh₃)₄, DMF, 16 h, 100 °C, 86%; (d) LiOH, THF, 1 h, rt, quantitative; (e) BOP, Et₃N, THF, 19 h, rt, 74%; (f) Raney Ni, 4.8 atm H₂(g), NH₃/MeOH, 21 h, rt, 99%; (g) HCl/dioxane, MeOH, 2–6 h, rt, 40%-quantitative; (h) 1,3-bis (tert-butoxycarbonyl)-2-methyl-2-thiopeudourea, HgCl₂, Et₃N, THF, 2.5 h, rt, 36%, (i) Cl-SO₂CH₃, pyridine, CH₂Cl₂, 16 h, rt, 50%.
Cyclohexyl analog 14 was prepared as shown in Fig. 7. (S)-3-methyl-1-(4-phenylpiperidin-1-yl)butan-2-amine IV-1, prepared according to previously reported methods15 was hydrogenated to afford the corresponding cyclohexyl intermediate IV-2. Routine BOP-mediated amidation of IV-2 with carboxylic acid I-1, followed by HCl deprotection furnished 14.

For the synthesis of indolinone analog 15 (Fig. 8), a reductive amination approach with 1-(piperidin-4-yl)-2,3-dihydro-1H-indol-2-one V-131 and commercially available Boc-L-valinal V-3 was utilized, rather than an amidation-deprotection-reduction sequence previously used 15, to avoid reduction of the indolinone lactam, decreasing the synthesis by one step and avoiding use of tedious borane. Trifluoroacetic acid deprotection of the reductive amination product then afforded intermediate V-4. Coupling of amine V-4 with carboxylic acid I-1 using propylphosphonic anhydride (T3P®), followed by HCl deprotection furnished 15. Similar methodology was also used to access indoline 16, starting from commercially available 1-(piperidin-4-yl)-2,3-dihydro-1H-indole V-2. Reductive amination with Boc-valinal V-3 afforded the iminium intermediate, which was then reduced with NaCNBH 3 in trifluoroethanol 32. Deprotection with trifluoroacetic acid then afforded amine V-5. Subsequent BOP-mediated coupling of amine V-5 with carboxylic acid I-1, followed by HCl deprotection gave 16.

**In vitro pharmacological characterization.** Compounds 2–16 were characterized in vitro for their binding affinities, intrinsic activity and antagonist potencies at the NOP, KOP, MOP and DOP receptors and compared to AT-076 (1) which was characterized in the same assays. Binding affinities of 1–16 at NOP, KOP, MOP and DOP were evaluated in radioligand competition experiments in membranes from CHO cells stably transfected with the respective human receptors, using the appropriate radioligands [3H]N/OFQ, [3H]U69,593, [3H]DAMGO and [3H]Cl-DPDPE, respectively, as described in the Methods 15,31,33,34. Concentrations of the analogs showing 50% inhibition of radioligand binding (IC50) were determined from concentration-response curves and binding affinities reported as Ki (nM) (Table 1) calculated from the Cheng-Prusoff equation, as described in the Methods. The intrinsic activity of the compounds was determined by their ability to stimulate [35S]GTPγS binding to cell membranes in a six-point concentration curve up to 10 μM and compared to the standard agonists N/OFQ (NOP), DAMGO (MOP), U69,593 (KOP), and DPDPE (DOP), conducted as described in Methods 31,34–36. None of the analogs tested had any intrinsic activity in the GTPγS assay at the four receptor subtypes.

For compounds whose binding affinity Ki was <50 nM, the antagonist potencies (pA2) were determined in the [35S]GTPγS functional assay using Schild analysis, where the shift in EC50 in the dose-response curve of the respective standard agonist is determined in the presence of at least 4 concentrations of the test antagonist. The pA2 values obtained in these analyses are shown in Table 2.

**Results**

To explore binding orientations of 115 at the opioid receptors with an SAR analysis, we replaced the 7-hydroxy of the Tic moiety of 1 with positively charged aminergic substituents, as in analogs 3–7, which, we hypothesized,
may interact with anionic residues in the EL2 loops of the NOP and KOP receptors. The uncharged nitrile analog 2 was also synthesized to explore the importance of the 7-OH in the Tic moiety. To our surprise, these modifications decreased binding affinity at all four receptors compared to that of the lead AT-076 (Table 1). The drop in affinity at NOP was particularly pronounced, over three orders of magnitude for 3–7 (see Table 1). At the KOP, MOP and DOP receptors, the effect of these modifications was less pronounced. The polar but uncharged nitrile analog 2 showed only a 6-fold drop in KOP affinity, but charged substituents as in analogs 3 and 4 caused a >100-fold decrease in binding affinity at KOP, MOP and DOP receptors. Replacing the entire Tic-OH moiety with lysine (6) or arginine (7) significantly decreased affinity for all four receptors.
Structural modifications at the opposite end of the molecule, i.e. replacing the 3-hydroxyl group of the phenylpiperidine with an amino group (10), interestingly increased NOP binding affinity 2-fold compared to the uncharged cyano precursor (8), giving a K_i of 30.72 ± 14.5 nM. The arginine analog 12, has even higher NOP affinity (K_i of 6.04 ± 1.32 nM), comparable to that of 1 at NOP. These modifications retained the high binding affinity at KOP and MOP but not the DOP receptors. Overall, the replacements of the 3-OH group were less detrimental to the affinity at NOP, and resulted in equi-potent binding affinity at KOP and MOP (analogs 9, 10 and 12). The importance of the 7-OH group in the Tic moiety was further confirmed with analogs 9, 11 and 13, because removal of this group significantly dropped affinity at all receptors, compared to 8, 10 and 12 respectively.

To further investigate the binding orientation of AT-076 at the NOP receptor, the 3-hydroxyphenyl group of AT-076 was replaced with hydrophobic moieties such as a cyclohexyl ring (14), indolinone (15) and indoline (16). Interestingly, these analogs show a significant decrease in binding affinity at the NOP receptor. However, at the KOP receptor, analogs 14 and 16 have sub-nanomolar affinity, being about 2–4-fold higher affinity than AT-076 itself. There was a modest decrease in affinity at MOP and DOP for these compounds compared to AT-076. With this enhancement of binding affinity at the KOP receptor, compounds 14 and 16 are selective KOP ligands, showing greater binding selectivity for KOP over MOP (11-fold for 14, 20-fold for 16), DOP (49-fold for 14, 370-fold for 16) and NOP (333-fold for 14, >1000-fold for 16) receptors, compared to the KOP antagonist JDTic, which shows only a 4-fold binding selectivity over MOP, 25-fold over DOP and 39-fold over NOP as determined in our experiments (Table 1).

Incorporation of a carbonyl group on 16 (to indolinone 15) reduces KOP affinity by 10-fold, and shows decreased affinity at MOP and DOP compared to 16.

Functional characterization of intrinsic (agonist) activity and antagonist potencies of the analogs was conducted using the [35S]GTP-S binding assay. As expected, none of the analogs had any agonist activity at any of the opioid receptors. On the other hand, several analogs that had nanomolar binding affinities for any of the opioid receptors also showed significant antagonist potencies at that receptor, reported as pA2 values shown in Table 2. Notably, compound 12, which has single digit nanomolar binding affinity at NOP and KOP and MOP (Table 1) also has high antagonist potency at these receptors (pA2 values 8.3, 9.8 and 9.2 respectively, Table 2). The subnanomolar binding affinity and high selectivity for KOP observed with analogs 14 and 16 also translate to high antagonist potencies at KOP for these compounds (pA2 = 10.5 and = 10.6, Table 2). Compounds 14 and 16 are therefore selective and potent KOP antagonists.

Discussion

The nanomolar binding affinity of AT-076 (1) at all four opioid receptors suggests that it has a chemotype that binds in the opioid binding pocket of all four opioid receptors, NOP, MOP, KOP and DOP15. 1 may possess a common opioid pharmacophore and can be used as a tool compound to probe ligand recognition features at all opioid receptors. Such information is useful for the design of multifunctional or selective opioid ligands, as needed, based on this scaffold. To aid such studies, we continued our SAR studies of 1 and investigated several chemical structure modifications, designed to inform the SAR at all four opioid receptors.

We previously reported the results of docking compound 1 in the NOP receptor crystal structure bound to antagonist C-24 (PDB No: 4EA3)15. 1 was found to bind in an extended conformation to NOP15, similar to the co-crystallized NOP antagonist C-2418. The piperidine nitrogen of 1 formed a salt bridge with the conserved Asp13013,32, a key interaction for ligands at all four opioid receptors. Similar to the benzofuran moiety of C-24, the 3-hydroxyphenyl moiety of 1 at the 4-position of the central piperidine ring, was oriented toward the intracellular end of the NOP binding site in a lipophilic pocket, comprised of Tyr13113-31, Met13413-36 and Trp27648. The opposite side of the molecule, i.e. the Tic-OH moiety, of AT-076 in this docked pose was oriented toward the extracellular end of the binding cavity towards the EL2 loop, enriched with anionic residues such as Glu196, Glu197 and Glu199 in NOP15,37. However, the SAR of analogs 2–7 showed a large drop in NOP receptor binding affinity when the 7-OH group of the Tic-OH moiety was replaced with positively charged groups designed to interact with these anionic residues of the EL2 loop (Table 1 and Fig. 2). On the other hand, similar modifications of the
| Cmpd | Structure | NOP     | KOP     | MOP     | DOP     |
|------|-----------|---------|---------|---------|---------|
| 1 (AT-076) | ![Structure](image1) | 1.75 ± 0.74 | 1.14 ± 0.63 | 1.67 ± 0.6 | 19.6 ± 1.3 |
| 2 | ![Structure](image2) | 1890 ± 270 | 6.73 ± 1.42 | 81.7 ± 12.4 | 73.7 ± 57.9 |
| 3 | ![Structure](image3) | 5540 ± 1150 | 118 ± 29 | 1490 ± 220 | 1566 ± 1133 |
| 4 | ![Structure](image4) | 3260 ± 780 | 129 ± 60 | 180 ± 70 | >10K |
| 5 | ![Structure](image5) | 7910 ± 1470 | 295 ± 65 | 4400 ± 2350 | >10K |
| 6 | ![Structure](image6) | >10K | 1518.4 ± 418.8 | >10K | >10K |
| 7 | ![Structure](image7) | >10K | 125.34 ± 2.4 | 1442.1 ± 57.6 | >10K |
| 8 | ![Structure](image8) | 61.32 ± 14.6 | 3.86 ± 0.09 | 2.93 ± 0.67 | 135.92 ± 59.7 |
| 9 | ![Structure](image9) | >10K | 542.9 ± 62.2 | 972.91 ± 20.95 | >10K |
| 10 | ![Structure](image10) | 30.72 ± 14.5 | 1.08 ± 0.01 | 0.68 ± 0.28 | 133.45 ± 24.9 |
| 11 | ![Structure](image11) | 202.14 ± 55.6 | 408.3 ± 30.8 | 284.0 ± 75.3 | >10K |
| 12 | ![Structure](image12) | 6.04 ± 1.32 | 2.11 ± 0.52 | 5.67 ± 2.38 | 53.61 ± 7.12 |

Continued
3-OH of the phenylpiperidine moiety retained NOP binding affinity similar to that of AT-076 (compound 12). SAR of analogs 2–7 suggests that AT-076 may not bind in the same orientation as the co-crystallized NOP antagonist C-24 as previously suggested by our docking results. On the other hand, the high affinity of compounds 10 and 12 at NOP suggests that the positively charged moiety(ies) replacing the 3-OH of the phenylpiperidine instead, may likely contribute to the high affinity by interacting with the negatively charged residues near the extracellular end and EL2 loop of the NOP binding pocket. This SAR supports a reversed binding mode than previously proposed, such that the 3-hydroxyphenyl on the piperidine ring is oriented towards the extracellular end of the receptor.

Table 1. Binding affinity of 1 (AT-076) analogs at the four opioid receptors, determined in competition radioligand displacement assays in cloned human opioid receptor-transfected cells. *Ki values were determined by competitive displacement of the respective radioligands—[^3H]N/OFQ–NOP,[^3H]U69,593–KOP,[^3H]DAMGO–MOP and[^3H]DPDPE–DOP receptor. The Ki was calculated from the IC50 values determined from the binding curves, using the Cheng–Prusoff equation. Values are the Mean ± SEM of at least three independent experiments run in triplicate.

| Cmpd | Structure | NOP (nM) | KOP (nM) | MOP (nM) | DOP (nM) |
|------|-----------|----------|----------|----------|----------|
| 3    |           | 43.32 ± 19.1 | 56.14 ± 5.38 | 408.33 ± 37.3 | >10 K |
| 4    |           | 84.4 ± 13.6 | 0.253 ± 0.015 | 2.79 ± 0.28 | 12.4 ± 8.0 |
| 5    |           | 91.19 ± 25.4 | 2.6 ± 1.13 | 19.08 ± 6.35 | 249.43 ± 31.0 |
| 6    |           | 566 ± 129 | 0.371 ± 0.096 | 7.68 ± 0.52 | 137.6 ± 45 |

Table 2. Antagonist potencies (pA2 ± SEM) determined by Schild analysis in functional assays measuring inhibition of agonist-induced[^35S]GTPγS binding at the four opioid receptors. pA2 values are given as mean ± SEM of at least two experiments performed in triplicate on two separate days. *ND = antagonist potency was not determined for compounds whose binding affinity was >50 nM. #N/C = compound showed a noncompetitive profile in Schild analysis. §pA2 value from a single experiment done in triplicate.

| Cmpd | NOP (pA2 ± SEM) | KOP (pA2 ± SEM) | MOP (pA2 ± SEM) | DOP (pA2 ± SEM) |
|------|-----------------|-----------------|-----------------|-----------------|
| 1    | 7.52 ± 0.13     | 8.366 ± 0.012   | 9.24 ± 0.012    | 7.57 ± 0.012    |
| 2    | ND              | N/C             | ND              | ND              |
| 8    | ND              | 9.57 ± 0.18     | 8.09 ± 0.11     | ND              |
| 10   | 6.88 ± 0.63     | 9.42 ± 0.13     | 7.81 ± 0.01     | ND              |
| 12   | 8.30 ± 0.33     | 9.8 ± 0.8       | 9.16 ± 0.52     | ND              |
| 13   | N/C             | N/C             | ND              | ND              |
| 14   | ND              | 10.46 ± 0.0010  | 9.16 ± 0.11     | 7.3738 ± 0.31   |
| 15   | ND              | 9.19 ± 0.16     | 7.98 ± 0.16     | ND              |
| 16   | ND              | 10.56 ± 0.31    | 8.32 ± 0.01     | ND              |
| SB-612111 | 9.28 ± 0.21 | ND              | ND              | ND              |
NOP binding pocket, rather than towards the intracellular end, as previously found in the docked pose of 15. Such an orientation would place the 7-hydroxy-4-(3-hydroxyphenyl)-1-piperidinyl)methyl}-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide in the hydrophobic pocket, lined with residues conserved among the four opioid subtypes such as Met1343.36. SAR showing poor NOP binding affinities for analogs 14 and 16, bearing hydrophobic replacements of the 3-hydroxyphenyl moiety, further suggests that these groups at the C-4 position of the central piperidine ring are likely oriented towards the polar extracellular end of the NOP binding pocket, supporting a flipped orientation of AT-076 analogs compared to co-crystallized antagonist C-24 at the NOP receptor.

Our SAR results suggest that NOP ligands of a chemotype different from the co-crystallized ligand C-24 may possibly bind in a different orientation than the co-crystallized ligand. Indeed, docking studies of other piperidine-based NOP antagonists J-113397 and its analog Trap-101 in the NOP crystal structure conducted by Miller et al.24 showed that these antagonists favored the ‘flipped’ orientation, in which the piperidine C-4 heterocycle is oriented towards the extracellular end of the binding pocket, whereas the piperidine N-1 cyclooctyl substituent is buried in the intracellular hydrophobic pocket. SAR studies such as reported here are therefore useful for investigating possible binding orientations of ligand chemotypes different than the co-crystallized ligands in the opioid receptor crystal structures.

At the KOP receptor, the high KOP selectivity and antagonist potency of analogs 14 and 16 suggests that these compounds likely bind to the KOP receptor in an orientation similar to that of JDTic in the KOP receptor crystal structure17. The 3-hydroxyphenyl ring of JDTic is situated in a pocket comprised of Val1182.63, Cys1313.25, Val1343.28 and Leu1353.29 (See Fig. 9). These residues could likely provide strong hydrophobic interactions for the cyclohexyl and indoline group replacements in 14 and 16, respectively, which may explain their high binding affinity. Docking the selective KOP antagonists 14 and 16 in the KOP crystal structure 4DJH17 confirmed this binding orientation similar to that of JDTic, in which the cyclohexyl and indoline groups of in 14 and 16 occupy the same pocket as the 3-hydroxyphenyl ring of JDTic, as shown in Fig. 9.

Conclusions

In summary, this SAR study of 1 reveals several interesting trends–(i) 1 represents an universal opioid antagonist chemotype that is not a morphinan scaffold. (ii) The 7-hydroxy-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide appears to be important pharmacophore for binding at all four opioid receptors since modifications in this moiety (2–7, 9, 11 and 13) causes significant loss of affinity at all four receptors. (iii) Substituents at the 4-position of the piperidinyl ring may be used to modulate affinity and selectivity, particularly for the KOP receptor. Such modifications resulted in the discovery of a selective KOP antagonist 16 from a pan antagonist lead compound 1. (iv) The SAR for 1 and its analogs at the NOP receptor highlights the limitations of docking using the X-ray crystal structures as a single tool for rational drug design. Rather, a combination of experimental SAR and docking allows for an accurate understanding of ligand recognition of structurally diverse ligands at the opioid receptors.
Methods

Thin layer chromatography was performed on Analtech silica gel GF 250 micron TLC plates. The plates were visualized with a 254 nm UV light and staining with iodine. Flash chromatography was carried out on F60 silica gel, 230–400 mesh, 60 Å (Silicycle SiliaFlash). NMR was recorded on a Varian Mercury Plus NMR (300 MHz), Varian Mercury 300 (300 MHz), or Varian 400 (400 MHz) using CDCl₃ or MeOD-d₄. Mass spectra were obtained on a LCQ Fleet Ion Trap LC/MS®, a micromass ZMD 1000 or PE Sciex API 150EX LC/MS using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) mode. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. HRMS analyses were performed by the Mass Spectrometry Service Laboratory, University of Minnesota Department of Chemistry, Minneapolis, MN on a Bruker BioTOF II HRMS using ESI mode. For final compounds 6 and 7, HPLC analysis was performed on a reverse phase Varian C18 column (2.0 × 50 mm), using a binary gradient of 95:5 solvent A (H₂O + 0.1% formic acid): solvent B (ACN) → 0:100 for 15 minutes, at a flow rate of 200 µL/min. Eluted peaks were monitored at 254 nm with a Finnigan Surveyor PDA Plus detector. For all remaining final compounds, HPLC analysis was performed on a reverse phase Agilent Zorbax SB-Phenyl column (5 µm, 2.1 × 150 mm), using a binary gradient of 95:5 solvent A (95/5 H₂O/ACN + 0.1% formic acid): solvent B (5/95 H₂O/ACN + 0.1% formic acid) → 0:100 for 10 minutes, at a flow rate of 0.4 mL/min. Eluted peaks were monitored at 254 nm with a Shimadzu SPD-10AV UV-Vis detector. All final compounds tested were confirmed to be of > 95% purity by the HPLC method described above.

General Procedure 1: Conversion of phenol to aryl triflate. To a stirred solution of the appropriate phenol intermediate (1.00 equiv) in THF (0.10 M) was added N-phenyl-bis(trifluoromethanesulfonimide) (3.00 equiv) and K₂CO₃ (8.00 equiv) under Ar(g), and the mixture was stirred at room temperature for 18–26 h. The reaction was diluted with H₂O and CH₂Cl₂. The layers were separated, and the aqueous solution was extracted 2X with CH₂Cl₂. The combined organic layers were washed with satd. NaCl(aq). NaCl(aq), dried over Na₂SO₄, filtered and concentrated. The crude residue was purified via flash chromatography (for I-2: Hexane/EtOAc 95/5 → 40/60, for I-3a: Hexanes/EtOAc 90/10 → 40/60, for III-3a: Hexanes/EtOAc 90/10 → 40/60, to afford the desired material.

General Procedure 2: Pd-catalyzed cyanation of aryl triflates. To a stirred solution of the appropriate triflate intermediate (1.00 equiv) dissolved in DMF (0.10–0.13 M) was added ZnCl₂ (1.00–1.70 equiv), and Ar(g) was bubbled through the mixture for 15 min. Pd(PPh₃)₄ (0.11–0.2 equiv) was added, and the mixture was heated at 80–100 °C for 1–16 h. The reaction was diluted with H₂O and EtOAc. The layers were separated, and the aqueous solution was extracted 2X with EtOAc. The combined organic layers were washed with satd. NaCl(aq), dried over Na₂SO₄, filtered and concentrated. The crude residue was purified via flash chromatography (for I-2: Hexanes/EtOAc 95/5 → 75/25, for III-3a: Hexanes/EtOAc 90/10 → 40/60, to afford the desired material.

General Procedure 3: Amidation. **Method A:** To a stirred solution of amine (1.00 equiv) in THF (0.1 M) was added the appropriate carboxylic acid (1.20 equiv), BOP (1.20 equiv) and Et₃N (5.00 equiv), and the reaction was stirred at room temperature for 2–23 h. The reaction was diluted with EtOAc and satd. NaHCO₃(aq). The layers were separated, and the aqueous solution was extracted 2X with EtOAc. The combined organic layers were washed with satd. NaCl(aq), dried over Na₂SO₄, filtered and concentrated. The crude residue was purified via flash chromatography (for I-3: Hexanes/EtOAc 95/5 → 75/25, for III-4a: Hexanes/EtOAc/NH₄OH(aq) 95/5/1 → 50/50/1, for III-4b: Hexanes/EtOAc/NH₄OH 90/10/1 → 40/60/1) to afford the desired material.

General Procedure 4: Boc deprotection. **Method A:** To a solution of Boc-protected intermediate (1.00 equiv) in CH₂Cl₂, was added TFA (1:1 or 1:2 v:v, 0.02–0.05 M), and the reaction was stirred at room temperature for 1–5.5 h. **Method B:** To a solution of Boc-protected intermediate (1.00 equiv) in CH₂Cl₂ (0.1 M) was added TFA (8–10 equiv), and the reaction was stirred at room temperature for 2.5–4 h. **Method C:** To a solution of Boc-protected intermediate (1.00 equiv) in MeOH (0.12–0.21 M) was added 4 M HCl/dioxane (55–165 equiv), and the reaction was stirred at room temperature for 1–11 h. The reaction was then concentrated to a solid.

General Procedure 5: Nitrile reduction. Cyano intermediate (1.00 equiv) was dissolved in 7 N HCl/MeOH (0.04–0.31 M), and Raney Nickel (75–100 wt % of substrate) was added. The mixture was hydrogenated at 4–4.8 atm H₂(g) for 5.5–21 h at room temperature, then filtered through a pad of Celite and concentrated. The residue was purified by flash chromatography (for III-5a: CH₂Cl₂/MeOH/NH₄OH(aq) 100/0/0 → 90/10/5, for III-5b: CH₂Cl₂/MeOH/NH₄OH(aq) 100/0/0 → 20/80/1, for IV: CH₂Cl₂/MeOH/NH₄OH(aq) 100/0/0 → 93/6/1) to afford the desired material.
2-(tert-butyl) 3-methyl (R)-7-cyano-3,4-dihydroisoquinoline-2,3(1H)-dicarboxylate (I-2). To a solution of (3 R)-2-(tert-Butyloxy carbonyl)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (I-1) (0.97 g, 3.29 mmol) in acetone (33.0 mL) was added LiOH (138 mg, 3.29 mmol, 1.00 equiv) and dimethyl sulfate (313 μmol, 3.54 mmol) to afford 82 mg of the title material as a 3HCl salt in quantitative yield. 1H NMR (400 MHz, CDCl3) δ 7.41–7.47 (2 H, m), 7.24–7.26 (1 H, m), 5.19–5.20 (1 H, m), 4.74 (1 H, dd, J = 16, 4 Hz), 4.49 (1 H, t, J = 20 Hz), 3.63 (3 H, app d, J = 4 Hz), 3.15–3.33 (2 H, m), 1.49 (9 H, app d, J = 28 Hz).

2-(tert-butyl) 3-methyl (R)-7-hydroxy-3,4-dihydroisoquinoline-2,3(1H)-dicarboxylate (I-3). Prepared according to General Procedure 3 Method A using (S)-3-(1-(6-(3-hydroxyphenyl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (I-5). To a solution of (3 R)-2-(tert-Butoxy carbonyl)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (I-1) (0.97 g, 3.29 mmol) in acetone (33.0 mL) was added LiOH (138 mg, 3.29 mmol, 1.00 equiv) and dimethyl sulfate (313 μmol, 3.54 mmol) to afford 82 mg of the title material as a 3HCl salt in quantitative yield. 1H NMR (400 MHz, CDCl3) δ 7.41–7.47 (2 H, m), 7.24–7.26 (1 H, m), 5.19–5.20 (1 H, m), 4.74 (1 H, dd, J = 16, 4 Hz), 4.49 (1 H, t, J = 20 Hz), 3.63 (3 H, app d, J = 4 Hz), 3.15–3.33 (2 H, m), 1.49 (9 H, app d, J = 28 Hz).
3.36–3.43 (2 H, m), 3.17–3.26 (3 H, m), 3.06 (1 H, t, J = 2.19–2.33 (1 H, m), 1.86–2.05 (3 H, m), 1.03 (6 H, app t, J = 6.66 (1 H, d, J = 9 Hz), 4.47 (2 H, s), 4.30–4.42 (2 H, m), 4.24 (2 H, s), 4.12 (1 H, d, J = 9 Hz), 3.59 (1 H, br s), 3.36–3.43 (2 H, m), 3.17–3.26 (3 H, m), 3.06 (1 H, t, J = 12 Hz), 2.89 (3 H, s), 2.76–2.84 (1 H, m), 2.48–2.61 (1 H, m), 2.19–2.33 (1 H, m), 1.86–2.05 (3 H, m), 1.03 (6 H, app t, J = 6.6 Hz), MS(ESI) m/z 529.3 (M + H)+. Anal. Calcd. for C32H40N4O4S·2.00 HCl·2.00 H2O·0.60 Dioxane: C, 52.88; H, 7.42; N, 8.11; found: C, 52.57; H, 7.10; N, 7.88.

dibenzy1 (5S)-6-(((5S)-1-((4-(3-hydroxyphenyl)piriperidin-1-yl)-3-methylbutan-2-yl)amino)-6-oxohexane-1,5-diyl)dicarbamate (I-1). Prepared according to General Procedure 3 Method A using (S)-3-((2-amino-3-methylbutyl)piperidin-4-yl)phenol (I-4)15 (100 mg, 0.38 mmol) and N2,N6-Bis(benzyloxy carbonyl)-L-lysine (190 mg, 0.46 mmol) to afford 160 mg of the title material in 64% yield. 1H NMR (300 MHz, CDCl3) δ 7.27–7.40 (10 H, m), 7.14 (1 H, t, J = 5.7 Hz), 6.83 (1 H, s), 6.66–6.74 (2 H, m), 6.20 (1 H, s), 5.26 (1 H, d, J = 9 Hz), 5.06–5.09 (2 H, m), 4.93 (1 H, s), 4.54 (1 H, s), 4.02–4.07 (2 H, m), 3.97 (1 H, s), 3.54 (1 H, s), 2.95–3.23 (3 H, m), 2.70–2.73 (2 H, m), 1.43–2.40 (13 H, m), 1.22 (4 H, d, J = 4.5 Hz), 0.89–0.96 (6 H, m), MS(ESI) m/z 659.64 (M + H)+.

(S)-2,6-diamo-N-((S)-1-((4-(3-hydroxyphenyl)piriperidin-1-yl)-3-methylbutan-2-yl)hexanamide (6). To a solution of intermediate II-1 (140 mg, 0.21 mmol) in THF (3.54 mL) was added Pd/C, 10% (35 mg), and the reaction was stirred at room temperature for 6 h under an atmosphere of H2(g). The solution was filtered through a pad of Celite and concentrated to afford 83 mg of the title material in quantitative yield, which was converted to the HCl salt by addition of 2 M HCl/ether. 1H NMR (300 MHz, CDCl3) δ 7.08 (1 H, t, J = 6 Hz), 6.58–6.69 (3 H, m), 3.96–4.02 (1 H, m), 3.37 (1 H, t, J = 6 Hz), 3.15 (1 H, d, J = 12 Hz), 2.88–3.01 (3 H, m), 2.40–2.52 (3 H, m), 2.26 (1 H, td, J = 12, 3 Hz), 2.07 (1 H, td, J = 12, 3 Hz), 1.48–1.88 (11 H, m), 0.94 (6 H, app t, J = 6 Hz). HRMS (ESI) Calcd for C32H36N4O4·2HCl·H2O·1/2 H2O: m/z 591.3061; found 591.3059. LCMS Rf = 0.78 min; m/z (M + H)+ = 391.38.

tert-butyl N-((1E)-6-(((4S)-4-(((tert-butoxycarbonyl)guanidino)methyl)-3-(((S)-1-((4-(3-hydroxyphenyl)piperidine-1-yl)-3-methylbutan-2-yl)carbamoyl)butyl)amino)carbonyl)(4S)-4-(((tert-butoxy)carbonyl)guanidino)methyl)carbamate (II-2). Prepared according to General Procedure 3 Method A using (S)-3-((2-amino-3-methylbutyl)piperidin-4-yl)phenol (I-4)15 (100 mg, 0.38 mmol) and Boc-Arg(Boc)-OH (217 mg, 0.46 mmol) to afford 260 mg of the title material in 95% yield. 1H NMR (300 MHz, CDCl3) δ 9.40 (1 H, s), 7.14 (1 H, t, J = 5.7 Hz), 6.76 (1 H, s), 6.69 (2 H, t, J = 5.7 Hz), 6.19 (1 H, d, J = 6 Hz), 5.45 (1 H, d, J = 6.3 Hz), 4.15 (1 H, q, J = 11.1, 6 Hz), 4.07–4.16 (2 H, m), 3.98–4.07 (2 H, m), 3.87–3.96 (1 H, m), 3.00–3.11 (1 H, m), 2.80–2.95 (2 H, m), 2.61–2.70 (1 H, m), 2.46–2.57 (3 H, m), 2.36–2.46 (2 H, m), 2.20–2.32 (1 H, m), 1.86–2.05 (3 H, m), 1.79 (1 H, s), 1.62–1.80 (7 H, m), 1.01–1.05 (6 H, m). LCMS Rf = 1.20 min; m/z (M + H)+ = 493.3. HRMS (ESI) Calcd for C36H50N4O6·2HCl·H2O·H2O·1/4 H2O: m/z 793.7 (M + H)+.
(S)-2-amino-5-guanidino-N-((S)-1-(4-(3-hydroxyphenyl)piperidin-1-yl)-3-methylbutan-2-yl)pentanamide (7). Prepared according to General Procedure 4 Method A from intermediate II-2 (250 mg, 0.35 mmol). The solution was concentrated, then triturated in diethyl ether overnight to afford 210 mg of the title material in 79% yield. 1H NMR (300 MHz, CDCl 3) δ 7.00 (1 H, d, J = 9 Hz), 6.61 (1 H, d, J = 9.3 Hz), 6.61 (1 H, br s), 5.85 (1 H, br s), 4.40–4.76 (3 H, m), 3.82 (1 H, br s), 3.28 (1 H, dd, J = 15.3 Hz), 3.00 (1 H, dd, J = 15.9 Hz), 2.71–2.82 (2 H, m), 2.36–2.50 (1 H, m), 1.88–2.22 (5 H, m), 1.64–1.72 (4 H, m), 1.51 (9 H, s), 0.96 (9 H, s), 0.87 (6 H, aff dd, J = 18.9 Hz), 0.17 (6 H, s). MS(ESI) m/z 784.8 (M + H) + 419.3129; found 419.3131. LCMS Rt = 0.79 min; m/z (M + H) + = 419.40.

(R)-2-(tert-butoxycarbonyl)-7-((tert-butyldimethylsilyl)oxy)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (III-1a). Prepared according to General Procedure 3 Method A using (S)-1-(2-amino-3-methylbut-2-yl)carbamoyl-4-phenol (I-4) (295 mg, 1.13 mmol) and intermediate III-1a (550 mg, 1.35 mmol) to afford 640 mg of the title material in 82% yield. 1H NMR (300 MHz, CDCl 3) δ 7.14 (1 H, t, J = 9 Hz), 7.05 (1 H, dd, J = 6 Hz), 6.66–6.72 (4 H, m), 6.60 (1 H, s), 6.34 (1 H, s), 5.98 (1 H, s), 4.79–4.82 (1 H, br s), 4.48–4.63 (2 H, m), 3.89 (1 H, br s), 3.04 (1 H, dd, J = 15.6 Hz), 1.66–2.79 (13 H, m), 1.51 (9 H, s), 0.96 (9 H, s), 0.87 (6 H, aff dd, J = 18.9 Hz), 0.17 (6 H, s). MS(ESI) m/z 885.7 (M + H) +.

tert-butyl (R)-7-((tert-butyldimethylsilyloxy)-3-((((S)-1-(4-(3-hydroxyphenyl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (III-2a). Prepared according to General Procedure 1 using intermediate III-2a (430 mg, 0.66 mmol) to afford 320 mg of the title material in 62% yield. 1H NMR (300 MHz, CDCl 3) δ 7.37 (1 H, t, J = 9 Hz), 7.22 (1 H, d, J = 9 Hz), 7.04–7.11 (3 H, m), 6.67 (1 H, dd, J = 9.3 Hz), 6.61 (1 H, br s), 5.85 (1 H, br s), 4.40–4.76 (3 H, m), 3.82 (1 H, br s), 3.28 (1 H, dd, J = 15.3 Hz), 3.00 (1 H, dd, J = 15.9 Hz), 2.71–2.82 (2 H, m), 2.36–2.50 (1 H, m), 1.88–2.22 (5 H, m), 1.64–1.72 (4 H, m), 1.51 (9 H, s), 0.94 (9 H, s), 0.85 (6 H, aff dd, J = 18.9 Hz), 0.17 (6 H, s). MS(ESI) m/z 784.8 (M + H) +.

tert-butyl (R)-7-((tert-butyldimethylsilyloxy)-3-((((S)-1-(4-(3-trifluoromethylsulfonyl)oxy)phenyl)piperidin-1-yl)butan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (II-3a). Prepared according to General Procedure 1 using intermediate II-3a (670 mg, 1.28 mmol) to afford 420 mg of the title material in 50% yield. 1H NMR (300 MHz, CDCl 3) δ 7.45 (1 H, t, J = 9 Hz), 7.32 (1 H, d, J = 5.4 Hz), 7.18–7.19 (6 H, m), 4.80–4.83 (2 H, m), 4.62 (2 H, d, J = 9.3 Hz), 3.85 (1 H, s), 3.17–3.26 (3 H, m), 2.85 (2 H, s), 2.41–2.53 (3 H, m), 1.70–1.74 (5 H, m), 1.51 (9 H, s), 0.83 (6 H, s). MS(ESI) m/z 654 (M + H) +.

tert-butyl (R)-7-((tert-butyldimethylsilyloxy)-3-((((S)-1-(4-(3-trifluoromethylsulfonyl)oxy)phenyl)piperidin-1-yl)butan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (III-3b). Prepared according to General Procedure 2 using intermediate III-3a (250 mg, 0.32 mmol) to afford 210 mg of the title material in 67% yield. 1H NMR (300 MHz, CDCl 3) δ 7.47–7.50 (2 H, m), 7.39–7.41 (2 H, m), 7.06 (1 H, d, J = 9.1 Hz), 6.68 (1 H, dd, J = 9.3 Hz), 6.60 (1 H, br s), 5.80 (1 H, br s), 4.40–4.79 (3 H, m), 3.81 (1 H, br s), 3.29 (1 H, dd, J = 15, 3 Hz), 3.01 (1 H, dd, J = 12, 3 Hz), 2.72–2.82 (2 H, m), 2.37–2.47 (1 H, m), 1.87–2.19 (5 H, m), 1.63–1.72 (4 H, m), 1.52 (9 H, s), 0.94 (9 H, s), 0.85 (6 H, aff dd, J = 21, 9 Hz), 0.15 (6 H, s). MS(ESI) m/z 661.5 (M + H) +.

tert-butyl (R)-7-((tert-butyldimethylsilyloxy)-3-((((S)-1-(4-(3-cyanophenyl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (III-4a). Prepared according to General Procedure 2 using intermediate III-3a (250 mg, 0.32 mmol) to afford 210 mg of the title material in 67% yield. 1H NMR (300 MHz, MeOD-d4) δ 7.43–7.57 (4 H, m), 6.97 (1 H, d, J = 6.6 Hz), 6.60–6.62 (2 H, m), 4.72 (1 H, br s), 4.52 (2 H, br s), 3.83 (1 H, br s), 3.00–3.18 (2 H, m), 2.82 (2 H, br s), 2.36–2.51 (3 H, m), 1.99–2.09 (2 H, m), 1.62–1.74 (5 H, m), 1.50 (9 H, s), 0.83 (6 H, d, J = 6 Hz). MS(ESI) m/z 547.2 (M + H) +. The title material was prepared according to General Procedure 4 Method A from the Boc-protected intermediate (55 mg, 0.101 mmol). The solution was concentrated to dryness, then partitioned between CH2Cl2 and satd. NaHCO3 (aq). The layers were separated, and the aqueous solution was extracted 2X with CH2Cl2. The combined organic layers were dried over Na2SO4 filtered and concentrated. The residue was purified via flash chromatography using
Prepared according to General Procedure 5 from intermediate III-3b (230 mg, 0.35 mmol) to afford 170 mg of the title material in 91% yield. 1H NMR (300 MHz, CDCl3) δ 7.39–7.52 (4 H, m), 7.14–7.21 (4 H, m), 5.85 (1 H, br s), 4.83–5.00 (1 H, m), 4.52–4.72 (2 H, m), 3.87 (1 H, br s), 3.41 (1 H, dd, J = 15.6, 3.6 Hz), 3.08 (1 H, dd, J = 15.6, 6 Hz), 2.65–2.90 (2 H, m), 2.11–2.46 (3 H, m), 2.01–2.30 (2 H, br s), 1.66–1.83 (6 H, m), 1.51 (9 H, s), 0.81 (6 H, br s), 0.17 (6 H, app d, J = 3 Hz). MS(ESI) m/z 665.5 (M + H)+. Anal. Calcd. for C27H38N4O2: C, 58.26; H, 7.68; N, 10.07; found: C, 57.94; H, 7.31; N, 9.91.

tert-butyl (R)-3-(((S)-1-(4-(3-cyanophenyl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl)-7-((tert-butyldimethylsilyl)oxy)-3,4-dihydroisoquinoline-2(1H)-carboxylate (III-6a). Prepared according to General Procedure 3 Method B from intermediate III-5a (76 mg, 0.14 mmol) in MeOH (1.00 mL) was added 2 M HCl in diethyl ether (1.00 mL), and the reaction was stirred at room temperature for 19 h. The reaction was concentrated, then triturated 3X with diethyl ether to afford 69 mg of the title material as a 3 HCl salt in 89% yield. 1H NMR (300 MHz, MeOD-d4) δ 7.07–7.44 (8 H, m), 6.67–6.69 (1 H, m), 4.28–4.80 (4 H, m), 4.56–4.72 (2 H, m), 3.84–3.94 (3 H, m), 2.68–2.79 (2 H, m), 2.37–2.40 (2 H, br s), 1.87–1.93 (2 H, m), 1.60–1.77 (6 H, m), 1.54 (9 H, s), 0.81 (6 H, br s), 0.81 (6 H, br s), 0.17 (6 H, app d, J = 3 Hz). MS(ESI) m/z 665.5 (M + H)+. Anal. Calcd. for C27H38N4O2·2HCl·1.7H2O·0.2 Diethyl Ether: C, 59.10; H, 7.39; N, 9.92; found: C, 59.20; H, 7.25; N, 9.76.
tert-butyl (R)-3-(((S)-1-(4-(((S,E)-4,9-bis((tert-butoxy carbonyl)amino)-13,13-dimethyl-3,11-dioxo-12-oxa-2,8,10-triazatetradec-9-en-1-yl)phenyl)piperidin-1-yl)-3-methylbutan-2-yl) carbamoyl)-7-hydroxy-3,4-tetrahydroisoquinoline-2(1H)-carboxylate (III-7a). Prepared according to General Procedure 3 Method B from intermediate III-6a (340 mg, 0.30 mmol) to afford 161 mg of the title material in 53% yield. 1H NMR (300 MHz, MeOD-d4) δ 7.72 (1 H, d, J = 6 Hz), 7.07–7.13 (3 H, m), 6.98 (1 H, d, J = 9 Hz), 6.60–6.63 (2 H, m), 4.71 (1 H, br s), 4.52 (2 H, br s), 4.37 (2 H, q, J = 15 Hz), 4.08 (1 H, br s), 3.84 (3 H, br s), 3.00–3.15 (2 H, m), 2.80 (2 H, br s), 1.93–2.39 (3 H, m), 1.64–1.79 (9 H, m), 1.43–1.52 (36 H, m), 0.83 (6 H, app d, J = 6 Hz). MS(ESI) m/z = 1008.2 (M + H)⁺.

tert-butyl (R)-3-(((S)-1-(4-(((S,E)-4,9-bis((tert-butoxy carbonyl)amino)-13,13-dimethyl-3,11-dioxo-12-oxa-2,8,10-triazatetradec-9-en-1-yl)phenyl)piperidin-1-yl)-3-methylbutan-2-yl) carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (III-6b). Prepared according to General Procedure 3 Method B from intermediate III-5b (120 mg, 0.22 mmol) to afford 102 mg of the title material in 78% yield, which was converted to the HCl salt by addition of 2 M HCl/ether. 1H NMR (300 MHz, MeOD-d4) δ 7.16–7.24 (2 H, m), 2.80–2.93 (6 H, m), 2.06–2.16 (4 H, m), 1.86–1.96 (3 H, m), 1.61–1.71 (2 H, m), 1.02 (6 H, app t, J = 6 Hz). HRMS (ESI) Calcd for C33H51N8O3 (M + H)⁺ 607.4052. LCMS Rt = 1.20 min; m/z (M + H)⁺ = 607.5.

(R)-N-(((S)-1-(4-(((S)-5-guanidinopentanamido)phenyl)piperidin-1-yl)-3-methylbutan-2-yl)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (12). Prepared according to General Procedure 4 Method A from intermediate III-7a (86 mg, 0.09 mmol). The solution was concentrated, then azeotroped 3X with diethyl ether to afford 60 mg of the title material in 93% yield. 1H NMR (300 MHz, MeOD-d4) δ 7.16–7.34 (8 H, m), 4.31–4.51 (6 H, m), 4.25 (1 H, dd, J = 12, 6 Hz), 4.07 (1 H, br s), 3.89 (1 H, t, J = 9 Hz), 3.63–3.66 (1 H, m), 3.06–3.23 (7 H, m), 2.81 (1 H, br s), 2.06–2.16 (4 H, m), 1.86–1.96 (3 H, m), 1.61–1.71 (2 H, m), 1.02 (6 H, app t, J = 6 Hz). HRMS (ESI) Calcd for C23H35N5O (M + H)⁺ 428.4. LCMS R_T = 1.20 min; m/z (M + H)⁺ = 428.4. Anal. Calcd. for C23H35N5O: C, 591.1; H, 8.3; N, 15.9. Found: C, 591.1; H, 8.4; N, 15.9.

(R)-N-(((S)-1-(4-(((S)-5-guanidinopentanamido)phenyl)piperidin-1-yl)-3-methylbutan-2-yl)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (13). Prepared according to General Procedure 4 Method B from intermediate III-6b (60 mg, 0.06 mmol). The solution was concentrated, then azeotroped 3X with diethyl ether to afford 60 mg of the title material as a 2 TFA salt in 93% yield. 1H NMR (300 MHz, MeOD-d4) δ 7.16–7.34 (8 H, m), 4.31–4.51 (6 H, m), 4.25 (1 H, dd, J = 12, 6 Hz), 4.07 (1 H, br s), 3.89 (1 H, t, J = 9 Hz), 3.63–3.66 (1 H, m), 3.06–3.23 (7 H, m), 2.81 (1 H, br s), 2.06–2.16 (4 H, m), 1.86–1.96 (3 H, m), 1.61–1.71 (2 H, m), 1.02 (6 H, app t, J = 6 Hz). HRMS (ESI) Calcd for C13H35N5O (M + H)⁺ 428.4. LCMS R_T = 1.20 min; m/z (M + H)⁺ = 428.4. Anal. Calcd. for C13H35N5O: C, 591.1; H, 8.3; N, 15.9. Found: C, 591.1; H, 8.4; N, 15.9.

(S)-1-(2-amino-3-methylbutyl)piperidin-4-ylindolin-2-one (V-4). To a solution of intermediate III-6a (340 mg, 0.30 mmol) to afford 161 mg of the title material in 78% yield, which was converted to the HCl salt by addition of 2 M HCl/ether. 1H NMR (300 MHz, MeOD-d4) δ 7.16–7.24 (2 H, m), 2.80–2.93 (6 H, m), 2.06–2.16 (4 H, m), 1.86–1.96 (3 H, m), 1.61–1.71 (2 H, m), 1.02 (6 H, app t, J = 6 Hz). HRMS (ESI) Calcd for C23H35N5O (M + H)⁺ 428.4. LCMS R_T = 1.20 min; m/z (M + H)⁺ = 428.4. Anal. Calcd. for C23H35N5O: C, 591.1; H, 8.3; N, 15.9. Found: C, 591.1; H, 8.4; N, 15.9.
(9.0 mL) was added Boc-L-valinal V-3 (754 mg, 3.75 mmol, 1.60 equiv) and HOAc (0.20 mL, 3.53 mmol, 1.50 equiv), and the reaction was stirred at room temperature for 0.5 h. NaBH(OAc)₃ (750 mg, 5.34 mmol, 1.50 equiv) was added, and the reaction was stirred at room temperature for 16 h. The reaction was diluted with CH₂Cl₂ and satd. NaHCO₃ was added. The layers were separated, and the aqueous solution was extracted 2X with CH₂Cl₂. The combined organic layers were washed with satd. NaCl(aq), dried over Na₂SO₄, filtered and concentrated. The crude residue was purified via flash chromatography with flash chromatography with Hexane/ EtOAc/NH₄OH(aq) 89/10/1 to afford 575 mg of tert-butyl (S)-(3-methyl-1-(4-(2-oxoindolin-1-yl)piperidin-1-yl)-7-hydroxy-3-(((S)-1-(4-(indolin-1-yl)piperidin-1-yl)-3-methylbutan-2-yl)carbamo-yl)-3,4-dihydroisoquinoline-2(1H)-carboxylate in 29% yield. 1H NMR (300 MHz, CDCl₃) δ 7.20–7.24 (2 H, m), 6.97–7.04 (3 H, m), 6.63–6.67 (1 H, m), 4.25–4.29 (1 H, m), 3.48 (2 H, s), 3.00 (2 H, dd, J = 16 Hz, 6 Hz), 2.93–3.00 (5 H, m), 2.02–2.49 (3 H, br s), 1.97–2.49 (3 H, br s), 1.65–1.87 (5 H, m), 1.51 (9 H, s), 0.81–0.89 (6 H, m). MS(ESI) m/z 402.4 (M+H)⁺.

(S)-1-(4-(indolin-1-yl)piperidin-1-yl)-3-methylbutan-2-amine (V-5). To a mixture of 1-(piperidin-4-yl)indoline 2,2,2-trifluoroacetate V-2 (582 mg free base equivalent, 2.88 mmol, 1.00 equiv) in 1,2-dichloroethane (15.0 mL) was added Et₃N (0.40 mL, 2.88 mmol, 1.00 equiv), and the reaction was stirred at room temperature for 5 min under Ar(g). To this mixture was added Boc-L-valinal V-3 (3.00 g, 8.63 mmol, 3.00 equiv) in 1,2-dichloroethane (33.0 mL), followed by HOAc (0.49 mL, 8.63 mmol, 3.00 equiv), and the reaction was stirred at room temperature for 1 h. NaBH(OAc)₃ (1.83 g, 8.63 mmol, 3.00 equiv) was added, and the reaction was stirred at room temperature for 16 h. The reaction was diluted with CH₂Cl₂ and satd. NaHCO₃(aq). The layers were separated, and the aqueous solution was extracted 2X with CH₂Cl₂. The combined organic layers were washed with satd. NaCl(aq), dried over Na₂SO₄, filtered and concentrated. The crude residue was purified via flash chromatography using Hexane/EtOAc/NH₄OH 90/10/0 to afford 95 mg of the title material as a 2 HCl salt in 98% yield. 1H NMR (free base, 400 MHz, CDCl₃) δ 7.19–7.24 (2 H, m), 6.97–7.04 (3 H, m), 6.63–6.67 (1 H, m), 4.25–4.29 (1 H, m), 3.48 (2 H, s), 3.00 (2 H, dd, J = 16 Hz, 6 Hz), 2.93–3.00 (5 H, m), 2.02–2.49 (3 H, br s), 1.97–2.49 (3 H, br s), 1.65–1.87 (5 H, m), 1.51 (9 H, s), 0.81–0.89 (6 H, m). MS(ESI) m/z 384.4 (M+H)⁺.

(R)-7-hydroxy-N-((S)-3-methyl-1-(4-(2-oxoindolin-1-yl)piperidin-1-yl)-7-hydroxy-3-(((S)-1-(4-(indolin-1-yl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxamide (15). To a solution of intermediate V-4 (771 mg, 1.46 mmol, 1.00 equiv) in 1,2-dichloroethane (15.0 mL) was added Et₃N (0.40 mL, 2.88 mmol, 1.00 equiv), and the reaction was stirred at room temperature for 10 min. (3 R)-2-(tert-Butoxycarbonyl)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid I-1 (470 mg, 1.60 mmol, 1.10 equiv) and proplyphosphonic anhydride (TPPO, 50 wt% in EtOAc) (2.60 mL, 8.74 mmol, 6.00 equiv) was added, and the reaction was stirred at room temperature for 25 h. The reaction was diluted with EtOAc and satd. NaHCO₃(aq). The layers were separated, and the aqueous solution was extracted 2X with EtOAc. The combined organic layers were washed with satd. NaCl(aq), dried over Na₂SO₄, filtered and concentrated to give 795 mg of crude tert-butyl (S)-1-(4-(indolin-1-yl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl-3,4-dihydroisoquinoline-2(1H)-carboxamide in quantitative yield. 1H NMR (300 MHz, CDCl₃) δ 7.02–7.07 (2 H, m), 6.62 (1 H, d, J = 6 Hz, 6.42 (1 H, d, J = 9 Hz), 4.67 (2 H, d, J = 9 Hz), 3.76 (2 H, dd, J = 9, 3 Hz), 3.35–3.58 (7 H, m), 2.96 (1 H, t, J = 6 Hz), 1.66–1.95 (5 H, m), 1.45–1.46 (9 H, m), 0.94 (6 H, app dd, J = 6 Hz). MS(ESI) m/z 388.4 (M+H)⁺. The title material was prepared according to General Procedure 4 Method B from the Boc-protected intermediate (770 mg, 1.99 mmol). The solution was concentrated, then azeotroped 3X with MeOH and 3X with CH₂Cl₂ to afford 1.04 g of the title material as a 2 TFA salt in quantitative yield, which was used directly in the next reaction. MS(ESI) m/z 288.3 (M+H)⁺.

(R)-7-hydroxy-N-((S)-1-(4-(indolin-1-yl)piperidin-1-yl)-3-methylbutan-2-yl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (16). Intermediate V-5 (392 mg, 1.36 mmol) and (3 R)-2-(tert-Butoxycarbonyl)-7-hydroxy-1,2,3,4-tetrahydroisoquinolines I-1 (480 mg, 1.64 mmol) were subjected to conditions described in General Procedure 3 Method A to afford 219 mg of tert-butyl (R)-7-hydroxy-3-(((S)-1-(4-(indolin-1-yl)piperidin-1-yl)-3-methylbutan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate in 29% yield. 1H NMR (300 MHz, CDCl₃) δ 6.98–7.07 (3 H, m), 6.60–6.68 (3 H, m), 6.40 (1 H, d, J = 9 Hz), 4.80 (1 H, br s), 4.49 (1 H, br s), 3.86 (1 H, br s), 3.22–3.35 (5 H, m), 2.91–3.00 (5 H, m), 1.97–2.49 (3 H, br s), 1.65–1.87 (5 H, m), 1.51 (9 H, s), 0.81–0.89 (6 H, m). MS(ESI) m/z 577.5 (M+H)⁺.
m/z 563.5 (M + H)⁺. The title material was prepared according to General Procedure 4 Method C from the Boc-protected intermediate (45 mg, 0.08 mmol). The crude residue was triturated with dioxane to afford 34 mg of the title material as a 2 HCl salt in 79% yield. ¹H NMR (300 MHz, MeOD-d₄) δ 7.08–7.36 (5 H, m), 6.75 (1 H, dd, J = 6, 3 Hz), 6.66 (1 H, d, J = 3 Hz), 4.12–4.36 (5 H, m), 3.83 (1 H, q, J = 7 Hz), 3.68 (3 H, br s), 3.34–3.40 (2 H, m), 3.05–3.23 (6 H, m), 2.69–2.85 (1 H, m), 2.41–2.52 (1 H, m), 2.01–2.11 (2 H, m), 1.83–1.90 (1 H, m), 1.01 (6 H, app t, J = 6 Hz). LCMS Rₜ = 1.29 min; m/z (M + H)⁺ = 463.3. HRMS (ESI) Calcd for C₂₈H₃₉N₄O₂ (M + H)⁺ 463.3067; found 463.3096.

**In vitro pharmacological Characterization.** Cells. Human NOP, mu, delta, and kappa opioid receptors were individually expressed in Chinese hamster ovary cells stably transfected with the human receptor cDNA, as we have described previously2,3. Kappa-CN cells were used for KOP radioligand binding assays, while Kappa-FLG19 cells were used in KOP [³⁵S]GTP-S functional assays. The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes.

Membrane preparation. The cell lines are grown to full confluency, then harvested for membrane preparation. The membranes are prepared in 50 mM Tris buffer (pH 7.4). Cells are scraped and centrifuged at 500 × g for 12 mins. The cell pellet is homogenized in 50 mM Tris with a Fisher Scientific PowerGen 125 rotor-stator type homogenizer, centrifuged at 20,000 × g for 25 mins, washed and recentrifuged once more and aliquoted at a concentration of 3 mg/mL protein per vial and stored in a −80 °C freezer till further use.

Receptor Binding. The assay is performed in a 96-well polysytle plate using triplicates of six concentrations of each test compound and tritiated ligands [³H]DAMGO (0.2 nM for MOP), [³H]DPDPE (0.2 nM for DOP), [³H]U69593 (0.2 nM for KOP). Nonspecific binding was determined by using 10 µM unlabeled nociceptin for NOP, 10 µM unlabeled DAMGO for MOP, 10 µM unlabeled DPDPE for DOP, and 10 µM unlabeled U69593 for KOP. Assays were initiated by addition of membrane homogenates and samples were incubated for 60 min at 25 °C in a total volume of 1.0 mL. In NOP receptor experiments, 1 mg/mL BSA is added to the assay buffer. The amount of protein in the binding assay was 15 μg. The incubation was terminated by rapid filtration through 0.5% PEI-soaked glass fiber filter mats (GF/C Filtermat A, Perkin-Elmer) on a Tomtec Mach III cell harvester and washed 5 times with 0.5 mL of ice-cold 50 mM Tris-HCl, pH 7.4 buffer. The filters were dried overnight and soaked with scintillation cocktail before counting on a Wallac Beta plate 1205 liquid scintillation counter. Radioactivity was determined as counts per minute (cpm). Full characterization of compounds includes analysis of the data for IC₅₀ values and Hill coefficients using GraphPad Prism. (ISI, San Diego, CA). Kᵢ values were determined by the method of Cheng and Prusoff39.

[³⁵S]GTP-S Functional assay. Functional assay is conducted in Buffer A, containing 20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl at pH 7.4. Membrane prepared as described above was incubated with [³⁵S]GTP-S (150,000 dpm/well), GDP (10 μM), and the test compound, in a total volume of 1 mL, for 120 minutes at 25 °C. Samples were filtered over Filtermat A and counted as described for the binding assays. A dose response curve with a prototypical full agonist at the respective receptor is conducted in each experiment to identify full and partial agonist compounds.

Determination of Antagonist potency. High affinity compounds (Kᵢ value < 50 nM) that demonstrate no agonist activity were evaluated for their antagonist potency by Schild analysis40, using an agonist full dose response curve in the presence of at least three concentrations of the test antagonist. pA₂ values and Schild slopes are determined using a statistical program designed for these experiments. If the Schild slope was found to be significantly different from −1.00, the antagonist activity was deemed non-competitive; in such cases, the pA₂ value is not reported. Equilibrium dissociation constants (Kᵢ values) were calculated as follows:

\[ Kᵢ = a/(DR − 1) \]

where “a” is the nanomolar concentration of the antagonist and “DR” is the ratio of the agonist EC₅₀ in the presence of a given concentration of antagonist.

Molecular Docking. Compounds were sketched and minimized using MMFF94 force field and charges in SybylX 1.2. Molecular docking was carried out using the Surflex-dock module in SybylX 1.2. The protomol was defined using the existing ligand JDTic inside the KOP receptor binding site of the KOP crystal structure (PDB ID: 4DJH). Docking was performed using the Geom protocol in Surflex-dock. A total of 20 poses were retained for each molecule. The top scoring poses of 14 and 16 were analyzed and compared to the bound ligand JDTic.

Data availability. The authors declare that all data supporting the findings of this study are available within the article.

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**Author Contributions**

N.T.Z. designed and supervised the research; V.B.J. designed and conducted the chemical synthesis and analyzed SAR data; W.E.P., E.W.T., J.L., conducted the pharmacological experiments and analyzed data; P.R.D. conducted the docking studies; V.B.J. and N.T.Z. wrote the manuscript.
Additional Information

Competing Interests: The authors declare that they have no competing interests.

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