Cyclic biphalin analogues with a novel linker lead to potent agonist activities at mu, delta, and kappa opioid receptors

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Abstract: In an effort to improve biphalin’s potency and efficacy at the μ-(MOR) and δ-opioid receptors (DOR), a series of cyclic biphalin analogues 1 – 5 with a cystamine or piperazine linker at the C-terminus were designed and synthesized by solution phase synthesis using Boc-chemistry. Interestingly, all of the analogues showed balanced opioid agonist activities at all opioid receptor subtypes due to enhanced κ-opioid receptor (KOR) activity. Our results indicate that C-terminal flexible linkers play an important role in KOR activity compared to that of the other cyclic biphalin analogues with a hydrazine linker. Among them, analogue 5 is a potent (Ki = 0.27, 0.46, and 0.87 nM; EC50 = 3.47, 1.45, and 13.5 nM at MOR, DOR, and KOR, respectively) opioid agonist with high efficacy. Based on the high potency and efficacy at the three opioid receptor subtypes, the ligand is expected to have a potential synergistic effect on relieving pain and further studies including in vivo tests are worthwhile.
Keywords:
Biphalin
Opioid receptors
MOR/DOR/KOR agonist
Synergistic analgesic effect
Cyclic peptides
Abbreviations:

Acm, acetamidomethyl; ACN, acetonitrile; BBB, blood brain barrier; Boc, t-butyloxycarbonyl; BOP, (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate); DAMGO, [DAla², NMePhe⁴, Gly-ol⁵]-enkephalin; DADLE, [DAla², DLeu⁵]-enkephalin; DMF, N,N-dimethylformamide; Dmt, 2,6-dimethyl tyrosine; DOR, δ-opioid receptor; EtOAc, ethyl acetate; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, hexafluorophosphate benzotriazole tetramethyl uronium; HEK, human embryonic kidney; HOBt, N-hydroxybenzotriazole; KOR, κ-opioid receptor; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; MeOH, methanol; MOR, µ-opioid receptor; NMM, N-methyl morpholine; Pen, penicillamine; RP-HPLC, reversed-phase high performance liquid chromatography; SAR, structure-activity relationship; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TLC, thin-layer chromatography
1. Introduction
Multifunctional ligands for the μ-opioid receptor (MOR) and δ-opioid receptor (DOR) have gained notoriety due to their promise in the realm of pain therapeutics [1]. It has been previously demonstrated that co-administration of MOR and DOR agonists lead to an attenuation of serious side effects caused by MOR agonists, such as tolerance, while augmenting potency and efficacy for the receptors [2-4]. Synergistic effects from MOR and DOR agonists have also been observed which increase analgesic efficacy and thereby allow the usage of lesser doses of MOR agonists and decrease unwanted MOR-related side effects [5].

![Figure 1. Design of new cyclic biphalin analogues](image)

Biphalin (Fig. 1) is a homodimer of an enkephalin-based tetrapeptide linked by hydrazine that shows high binding affinities in the nanomolar range at the MOR and DOR, and potent antinociceptive effects with promising bioavailability including potential blood brain barrier (BBB) penetration [6-10]. For these reasons, numerous structure-activity relationship (SAR) studies have been performed on biphalin to develop efficacious therapeutics for the treatment of pain, but most studies have been focused on modifications of the C-terminal linker and 2,2'- and/or 4,4'-amino acid residues [9,11-13]. Peptide cyclization is a very efficient tool to improve receptor specificity (lower off-target toxicity), potency, and efficacy of peptides along with proteolytic stability and bioavailability by limiting the peptide’s dynamic nature. Limiting conformational variants by constraints induced by formation of covalent bonds such as lactones, lactams, and disulfides, enhances their ability to interact with target receptors. Despite the benefits of incorporating cycles into peptides, few attempts have been made to cyclize biphalin’s structure [9,14-17]. In those cyclic biphalin studies, L-chirality of Cys and Pen residues at positions 2 and
resulted in a significant loss in affinity and efficacy at the opioid receptors (OR), whereas D-chirality enhanced affinity and efficacy, similar to previous SAR studies on linear analogues [15,16].

Aaa: Cys for 1 & 2, Pen for 3; Bbb: Tyr for 1 & 3; Dmt for 2. i) 2.2 equiv Bop/2.2 equiv HOBT/4.4 equiv NMM, 0 °C for 30 min & rt for 2-4 h ii) 95% TFA/5% TIS, 0 °C for 20-30 min

On the basis of these data, we sought to design and investigate a series of cyclic biphalin derivatives of which the homodimeric opioid pharmacophores are linked through a flexible cystamine or piperazine and 2,2′ positions are utilized for cyclization via a disulfide bond between DPen or DCys residues. Compared to constrained aromatic linkers, the flexible piperazine linker was shown to be more potent at the MOR and DOR, and therefore, another flexible linker, cystamine, was also chosen and utilized in this study. Additionally, Tyr residues at positions 1,1′ were substituted with 2,6-dimethyl-tyrosine (Dmt) residues in an effort to increase potency and
efficacy at the ORs [18,19]. As a result, a series of nonselective MOR/DOR/κ-opioid receptor (KOR) agonists were designed and synthesized (Fig. 1).

2. Results
The designed cyclic biphalin analogues were prepared by stepwise liquid phase peptide synthesis using the N\(^{\alpha}\)-Boc-chemistry approach (Scheme 1, Table 1). During the stepwise chain elongations, intermediate peptides were isolated by routine precipitation using common organic solvents, typically diethyl ether. A work-up process using basic (5% NaHCO\(_3\)) and subsequent acidic aqueous solutions (5% citric acid) removed excess amounts of unreacted N\(^{\alpha}\)-Boc-amino acids and coupling reagents from the reaction mixture and allowed intermediate peptides to be isolated by via precipitation. Cyclization of Acm-protected linear hexapeptides were performed using an I\(_2\) solution prior to N\(^{\alpha}\)-Boc-group deprotection due to sluggishness caused by N\(^{\alpha}\)-free amino groups. After cyclization, the cyclic N\(^{\alpha}\)-Boc-hexapeptides were deprotected and coupled with N\(^{\alpha}\)-Boc-Tyr-OH or N\(^{\alpha}\)-Boc-Dmt-OH. By following the synthetic scheme, we were able to accelerate disulfide bond formation and avoid serious side reactions caused by iodination of tyrosine or Dmt residues. In our first trial to cyclize a linear octapeptide using I\(_2\), the iodinated cyclic octapeptide was obtained as a major product. To avoid the unwanted side reaction, an N\(^{\alpha}\)-Boc-protected linear hexapeptide was cyclized first, and then coupled with N\(^{\alpha}\)-Boc-Tyr-OH or N\(^{\alpha}\)-Boc-Dmt-OH. After the final chain elongation and deprotection, crude products were isolated by preparative RP-HPLC to afford more than 98% purity of cyclic analogues 1 - 5 in overall 20-40% yields.

To evaluate an analogue’s biological activity, in vitro binding and functional assays were performed at the MOR, DOR, and KOR. Ki determinations, receptor binding profiles, and agonist functional data (EC\(_{50}\) and E\(_{\text{max}}\)) were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2013-00017-C (NIMH PDSP) [20-22].

Table 1. Analytical Data of Cyclic Biphalin Analogues

<table>
<thead>
<tr>
<th>Structure</th>
<th>Molecular Formula</th>
<th>(M + H)(^{+}) Calculated</th>
<th>(M + H)(^{+}) Observed</th>
<th>HPLC (t_R) (min)</th>
<th>aLogP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c(^2,2,2)(Tyr-nCys-Gly-Phe)(_2)-piperazine</td>
<td>C(<em>{6})H(</em>{16})N(<em>{10})O(</em>{3})S(_{2})</td>
<td>1025.4014</td>
<td>1025.40081</td>
<td>17.7</td>
</tr>
<tr>
<td>2</td>
<td>c(^2,2,2)(Dmt-nCys-Gly-Phe)(_2)-piperazine</td>
<td>C(<em>{5})H(</em>{16})N(<em>{10})O(</em>{3})S(_{2})</td>
<td>1081.4640</td>
<td>1081.46647</td>
<td>18.3</td>
</tr>
<tr>
<td>3</td>
<td>c(^2,2,2)(Tyr-nPen-Gly-Phe)(_2)-piperazine</td>
<td>C(<em>{5})H(</em>{16})N(<em>{10})O(</em>{3})S(_{2})</td>
<td>1081.4640</td>
<td>1081.46206</td>
<td>18.7</td>
</tr>
<tr>
<td>4</td>
<td>c(^2,2,2)(Tyr-nCys-Gly-Phe)(_2)-cystamine</td>
<td>C(<em>{6})H(</em>{16})N(<em>{10})O(</em>{3})S(_{4})</td>
<td>1091.3612</td>
<td>1091.35949</td>
<td>20.4</td>
</tr>
<tr>
<td>5</td>
<td>c(^2,2,2)(Dmt-nCys-Gly-Phe)(_2)-cystamine</td>
<td>C(<em>{5})H(</em>{16})N(<em>{10})O(</em>{3})S(_{4})</td>
<td>1147.4238</td>
<td>1147.42226</td>
<td>21.2</td>
</tr>
</tbody>
</table>

*FAB-MS (JEOL Hx110 sector instrument) or MALDI-TOF (Brucker Ultraflex III).

*Performed on a Hewlett Packard 1100 [C-18, Vydac, 4.6 mm x 250 mm x 5 μm, 10-100% acetonitrile containing 0.1% TFA within 45 min, 1 mL/min].

*http://www.vcclab.org/lab/alogps/ [23].
Table 2. Binding Data of Ligands 1-5\textsuperscript{a}

<table>
<thead>
<tr>
<th>Ligand</th>
<th>MOR [\textsuperscript{3}H]DAMGO\textsuperscript{b}</th>
<th>DOR [\textsuperscript{3}H]DADLE\textsuperscript{c}</th>
<th>KOR [\textsuperscript{3}H]U69593\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKi (nM)</td>
<td>Ki (nM)</td>
<td>pKi (nM)</td>
</tr>
<tr>
<td>1</td>
<td>8.05 ± 0.07</td>
<td>8.9</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>8.60 ± 0.08</td>
<td>2.5</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>8.54 ± 0.07</td>
<td>2.9</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>8.63 ± 0.07</td>
<td>2.4</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>9.57 ± 0.07</td>
<td>0.27</td>
<td>9.3 ± 0.1</td>
</tr>
<tr>
<td>Biphalin\textsuperscript{e}</td>
<td>12 ± 2</td>
<td>4.6 ± 0.2</td>
<td>270 ± 15</td>
</tr>
<tr>
<td>6\textsuperscript{f}</td>
<td>5.2 ± 0.5</td>
<td>1.9 ± 0.2</td>
<td>257 ± 25</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Membrane preparations were made from transient HEK cells expressing the appropriate receptor.

\textsuperscript{b}Kd = 1.73 ± 0.14 nM.

\textsuperscript{c}Kd = 1.85 ± 0.15 nM.

\textsuperscript{d}Kd = 1.07 ± 0.10 nM. 6: \textsuperscript{1}\textsuperscript{2}\textsuperscript{3}(Tyr-\textsuperscript{t}Pen-Gly-Phe-NH\textsubscript{2}).
Table 3. Functional Data of Ligands 1 - 5: Agonist Mode

<table>
<thead>
<tr>
<th></th>
<th>MOR</th>
<th></th>
<th>DOR</th>
<th></th>
<th>KOR</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>pEC\text{50}</strong></td>
<td><strong>EC\text{50} (nM)</strong></td>
<td><strong>E_{\text{max}} (%)</strong></td>
<td><strong>pEC\text{50}</strong></td>
<td><strong>EC\text{50} (nM)</strong></td>
<td><strong>E_{\text{max}} (%)</strong></td>
<td><strong>pEC\text{50}</strong></td>
<td><strong>EC\text{50} (nM)</strong></td>
<td><strong>E_{\text{max}} (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.29 ± 0.08</td>
<td>513</td>
<td>54</td>
<td>6.61 ± 0.86</td>
<td>245</td>
<td>77</td>
<td>6.47 ± 0.06</td>
<td>339</td>
<td>92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.95 ± 0.05</td>
<td>112</td>
<td>58</td>
<td>7.93 ± 0.06</td>
<td>117</td>
<td>90</td>
<td>7.20 ± 0.05</td>
<td>63.1</td>
<td>82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.72 ± 0.09</td>
<td>191</td>
<td>86</td>
<td>6.57 ± 0.04</td>
<td>269</td>
<td>206</td>
<td>6.57 ± 0.15</td>
<td>269</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7.06 ± 0.09</td>
<td>87.1</td>
<td>90</td>
<td>7.57 ± 0.06</td>
<td>26.9</td>
<td>91</td>
<td>6.87 ± 0.08</td>
<td>135</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.46 ± 0.14</td>
<td>3.47</td>
<td>119</td>
<td>8.84 ± 0.15</td>
<td>1.45</td>
<td>106</td>
<td>7.87 ± 0.12</td>
<td>13.5</td>
<td>98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)MOR, DOR, and KOR expressed from HEK cells.

\(^b\)Compared to DAMGO (pEC\text{50} = 8.94).

\(^c\)Compared to DADLE (pEC\text{50} = 7.36).

\(^d\)Compared to Salvinorin A (pEC\text{50} = 9.43).
Binding affinities at the ORs were determined for ligands 1 - 5 using membranes prepared from transient human embryonic kidney (HEK) cells. Competition binding assays were performed against radiolabeled ligands $[^3H]$DAMGO, $[^3H]$DADLE, and $[^3H]$U69593 for MOR, DOR, and KOR, respectively (Table 2). In these assays, interestingly, all ligands showed balanced binding affinities (selectivity < 8-fold) at the three subtype receptors due to enhanced KOR affinity ($K_i$: 0.87 - 19 nM). Biphalin interacts with MOR and DOR strongly but not with KOR ($K_i$ = 270 nM), and its cyclic analogue 6 with a disulfide bond at positions 2 and 2’ was shown to retain the same biological profile as biphalin [16]. Based on this previous SAR, the enhanced KOR binding affinity is considered to be attributed to linker modifications from a hydrazine to a piperazine or a cystamine.

Overall, ligands linked via a cystamine (4 and 5), which is a more flexible and dynamic motif, showed slight affinity improvements relative to ligands with a piperazine linker (1 and 2). Modification of Tyr residues at positions 1 and 1’ with Dmt also improved binding affinities at the ORs in ligands 2 and 5. Ligand 3 substituted dPen for dCys, a more constrained residue due to the presence of geminal methyl groups on $\beta$, and enhanced DOR binding affinity more so than that of MOR and KOR (14-fold at DOR vs 3-fold for both MOR and KOR). It was shown that a bulky disulfide bond and the geminal methyl groups of a Pen residue limit backbone flexibility and increase DOR activity in DPDPE. Ligand 3 might resemble the DPDPE structure and thus its increased DOR activity. It turned out that ligand 5 with Dmt at positions 1 and 1’ with a cystamine linker is the most potent lead, which displayed a subnanomolar range of binding affinities at the three ORs.

Opioid functional activity was explored by employing a split luciferase cAMP assay using HEK cells that stably express the respective OR (Table 3). For agonist modes, efficacies were compared to DAMGO, DADLE, and Salvinorin A at MOR, DOR, and KOR, respectively. Antagonist mode was run using naltrexone, naltrindole, and GNTI as reference ligands for the MOR, DOR, and KOR, respectively. Ligands 1 – 5 did not show antagonist activities at the ORs. Overall, ligands’ functional activities correlated with their binding affinities at the ORs, and most ligands exhibited high efficacy at the MOR, DOR, and KOR. Ligand 5, which showed binding affinities in the subnanomolar range at the ORs, was the most potent MOR/DOR/KOR agonist with the same high efficacies ($E_{max}$=119%, 106%, and 98% for MOR, DOR, and KOR, respectively) relative to the reference ligands. In these functional assays, it was again observed that the linkers piperazine and cystamine aided ligands 1-5 to interact with the KOR, and therefore, all ligands except for 3 achieved high efficacies ($E_{max}$: 82 - 99%) relative to Salvinorin. The ligands containing piperazine with dCys (1 and 2) exhibited lower efficacy at the MOR compared to the DOR and KOR (Table 3). Ligand 3, which incorporated the dPen residues as a bridge, turned out to be an extremely efficacious agonist at the DOR relative to DADLE ($E_{max}$ = 206%). Despite encouraging $E_{max}$ results, $EC_{50}$ values achieved low potency (240 nM). Ligands 4 and 5 gave more promising outcomes which had cystamine and dCys. Replacement of Tyr residues with Dmt in ligand 5
resulted in the discovery of a potent MOR/DOR/KOR agonist with a low nanomolar range of EC_{50} values (3.47 nM, 1.45 nM, and 13.5 nM at the MOR, DOR, and KOR, respectively) and high efficacies (119%, 106%, and 98% at the MOR, DOR, and KOR, respectively).

3. Discussion
A series of cyclic biphalin analogues 1 – 5 with a cystamine or piperazine linker at the C-terminus was designed and synthesized by solution phase synthesis using Boc-chemistry, which resulted in the discovery of potent MOR/DOR/KOR agonists. Both disulfide bridge and linker variants resulted in mixed opioid agonist profiles due to additional KOR agonist activity. It appeared that ligands which employed cystamine as a linker afforded more potent agonist profiles, whereas those with piperazine had attenuated effects and were non-selective. The series showed mostly nanomolar range of high affinities at KOR coupled with high agonist efficacies and mixed potencies. Considering that biphalin is MOR/DOR selective and our ligands demonstrated non-selective affinities and activities across all ORs, the recovery of KOR interaction may be attributed to the termini modifications to a cyclic structure. Based on the message-address concept of enkephalin, the N-terminal Tyr or Dmt residues are in the message region which correlate with the ligand’s efficacy, and the C-terminal linker is a part of the address region which modifies ligand selectivity [23,24]. It has been suggested that dynorphin, an endogenous KOR agonist, accomplishes KOR selectivity through several basic amino acids at the C-terminus, and enkephalin, a DOR agonist, can be modified at the C-terminus to acquire KOR activity and selectivity. Our SAR results seemed to support the explanation. Based on the biological profile of hydrazine linked cyclic analogue 6, improved KOR activities were considered due to cystamine and piperazine linkers in the address region. Apparently, the more flexible linear linker cystamine (4 and 5), was preferred to piperazine (1, 2, and 3) for all three subtype ORs. Introduction of the constrained Tyr derivative Dmt at positions 1 and 1’ enhanced opioid activities in ligands 2 and 5 as demonstrated in many previous studies. Ligand 5 was the most potent, non-selective agonist with high efficacies and affinities at the MOR, DOR, and KOR, but it is worthwhile to note that ligand 3 with bulky DPen residues at positions 2 and 2’ showed the highest agonist efficacy at the DOR. This suggests DPen’s ability to selectively augment receptor interaction at the DOR as shown in DPDPE.

4. Material and methods
All amino acid derivatives except Boc-Dmt-OH (RSP Amino Acid Analogues, Inc,) were purchased from NovaBiochem or AAPPTec. Coupling reactions were monitored via TLC with the following mobile phase: CHCl_3/MeOH/AcOH = 90:10:1 (vol/vol/vol) with ninhydrin spray used for detection. Analytical RP-HPLC was performed on a Hewlett Packard 1100 [C-18, Vydac, 4.6 mm x 250 mm, 5 µm, 10-100% of acetonitrile containing 0.1% TFA within 45 min, 1 mL/min] and preparative RP-HPLC on a Hewlett-Packard 1100 [C-18, Microsorb-MV, 10 mm x 250 mm,
10 µm]. Mass spectra were obtained using FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF. aLogP values were calculated by ALOGPS 2.1 program [25,26].

Ligands 1 - 5 were prepared via stepwise synthesis using Nα-Boc chemistry starting from either piperazine (1 - 3) or cystamine (4 and 5). Nα-Boc-Phe-OH (2.2 equiv), BOP (2.2 equiv), and HOBT (2.2 equiv) were dissolved in DMF and cooled in an ice bath for 10 min. NMM (4.4 equiv) was added dropwise to the reaction mixture and warmed to room temperature with additional stirring for 2-4 hours. After confirming the disappearance of the Nα-amino group by TLC, the mixture was diluted with 10-fold volume of EtOAc and washed with 5% NaHCO₃ (weight/vol) three times, 5% citric acid (weight/vol) two times, brine, and water, consecutively. The combined organic layers were dried under anhydrous Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure and the residual oil was triturated with diethyl ether to afford the corresponding Nα-Boc peptide as a white powder. The Nα-Boc group was deprotected by a TFA solution containing 5% TIS (vol/vol) at 0 °C for 20 - 30 minutes. Deprotection was monitored by TLC. After completion of chain elongation to the bridging amino acid residue (Boc-dPen(Acm)-OH or Boc-dCys(Acm)-OH), the Nα-Boc protected linear hexapeptides were subjected to a disulfide bond formation using I₂. A 0.06 M iodine solution was added dropwise to a linear hexapeptide solution (0.1 g peptide in 1 L of 20% MeOH in water (vol/vol)) until a light-yellow color persisted and left overnight. The progress of disulfide bond formation was monitored by analytical RP-HPLC. After completion of the disulfide bond, the reaction mixture was quenched using ascorbic acid and evaporated under reduced pressure. The concentrated reaction mixture was triturated with diethyl ether to give a Nα-Boc-protected cyclic hexapeptide as a powder. The formed powder was subjected to Nα-Boc-deprotection and final coupling with Nα-Boc-Tyr-OH or Nα-Boc-Dmt-OH. Final crude cyclic peptides were isolated by preparative RP-HPLC (20-70% ACN within 25 mins) to afford pure cyclic biphalin analogues as white powders in overall yields of 20 - 40%. Purity was validated as >98% by analytical HPLC (10-100% ACN in water containing 0.1 % TFA (vol/vol) in 45 min).

Radioligand binding competition assays and the split luciferase cAMP assays were performed in the Roth lab through the NIMH Psychoactive Drug Screening Program. Experimental details can be found at the PDSP website [20-22].

5. Conclusions

Cyclization of biphalin, a MOR/DOR agonist, along with a linker modification, resulted in the discovery of a highly potent MOR/DOR/KOR agonist (5), which is considered to have potential synergistic effects between the three opioid receptors. Our studies demonstrated that the replacement of Tyr residues at positions 1 and 1’, a common message region of the ORs, with Dmt residues, improved opioid potency at the ORs and linker modification at the C-terminus, the address region of the ORs, resulted in an increase in KOR activity. A synergistic analgesic effect is advantageous due to reducing necessary dosages to relieve pain and therefore attenuate opioid related side effects. However, utilizing KOR agonists to relieve pain is not typically suggested...
because of serious side effects such as dysphoria. Many studies evidenced that biased KOR agonists without β-arrestin recruitment can avoid such unwanted side effects. Since dysphoria is considered a side effect corresponding to the central nervous system, some studies also suggested that KOR agonists that do not penetrate the BBB but instead act with the peripheral nervous system could be another solution. Therefore, even with a concern regarding the exploitation of KOR agonists, having a balanced MOR/DOR/KOR agonist, if one can localize its action to the PNS, would benefit through efficient pain relief by dose reduction and thus avoiding serious side effects associated with the other ORs. From this point of view, ligand 5 exhibited therapeutic potential and further studies including in vivo tests are worthwhile.

Conflicts of Interest
The authors declare no conflict of interest.

Acknowledgments
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References and notes
22. The NIMH PDSP is directed by Bryan L. Roth at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. https://pdspdb.unc.edu/pdspWeb/