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Unexpected Opioid Activity Profiles of Analogues of the Novel Peptide Kappa Opioid Receptor Ligand CJ-15,208

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An alanine scan was performed on the novel κ opioid receptor (KOR) peptide ligand CJ-15,208 to determine which residues contribute to the potent in vivo agonist activity observed for the parent peptide. These cyclic tetrapeptides were synthesized by a combination of solid-phase peptide synthesis of the linear precursors, followed by cyclization in solution. Like the parent peptide, each of the analogues exhibited agonist activity and KOR antagonist activity in an antinociceptive assay in vivo. Unlike the parent peptide, the agonist activity of the potent analogues was mediated predominantly, if not exclusively, by μ opioid receptors (MOR). Thus analogues **2** and **4**, in which one of the phenylalanine residues was replaced by alanine, exhibited both potent MOR agonist activity and KOR antagonist activity in vivo. These peptides represent novel lead compounds for the development of peptide-based opioid analgesics.

Introduction

Recent reports suggest that κ opioid receptor (KOR) antagonists could have potential therapeutic application in the treatment of mood disorders and drug abuse.[1] Pretreatment with the nonpeptide KOR-selective antagonists nor-binaltorphimine (nor-BNI) or JDTic decrease immobility in the forced swim assay similar to antidepressants,[2] and have been shown to reduce behavioral measures of anxiety in rats.[3] Pretreatment with these antagonists also prevents stress-induced reinstatement of extinguished cocaine-seeking behavior.[2c,4] Likewise, heroin-dependent patients treated with a "functional KOR antagonist" (buprenorphine plus naltrexone to block μ opioid receptors (MOR)) for 12 weeks showed significantly improved drug abstinence relative to patients treated only with naltrexone.[5]

These nonpeptide antagonists demonstrate notably prolonged durations of activity,[1,6] antagonizing KOR for weeks after a single dose.[7] This unusual pharmacological profile can complicate their use as pharmacological tools and could conceivably slow their development for clinical use, sparking interest in shorter-acting KOR-selective antagonists.

We have a long-standing interest in peptide ligands for KOR, particularly those that demonstrate KOR-selective antagonism. A number of analogues of the endogenous opioid peptide dynorphin A have demonstrated KOR antagonism (for example, see reference [8]). Modifications to linear peptides can decrease proteolytic cleavage so that the peptide's activity is preserved after systemic administration. This was demonstrated for the KOR-selective peptide antagonist zyklophin ([*N*-benzyl-Tyr[1],*cyclo*(D-Asp[5],Dap[8])]dynorphin A-(1–11)NH_2) developed in our research group,[8e] which exhibits KOR-selective antagonist activity following systemic (subcutaneous) administration,[9] and prevents stress-induced reinstatement of extinguished cocaine-conditioned place preference after subcutaneous administration.[9]

The cyclic tetrapeptide CJ-15,208 was reported to preferentially bind to KOR and antagonize the activity of a KOR agonist in the rabbit vas deferens smooth muscle preparation,[10] but the stereochemistry of the Trp residue in this natural product was not determined. We therefore undertook the synthesis of both tryptophan isomers of this cyclic tetrapeptide,[11] and found that the optical rotation of the L-Trp isomer **1** is consistent with that reported for the natural product (Figure 1a). Although the L-Trp peptide did not exhibit any agonist activity at either KOR or MOR in vitro,[12] it unexpectedly exhibited robust agonist activity in vivo in the 55 °C warm-water tail-withdrawal antinociceptive assay in addition to KOR-selective antagonist activity.[12a]

Therefore we undertook structure–activity relationship (SAR) studies of CJ-15,208, first performing an alanine scan (Figure 1b) of the peptide to determine which amino acid side chains are important for interaction with opioid receptors and

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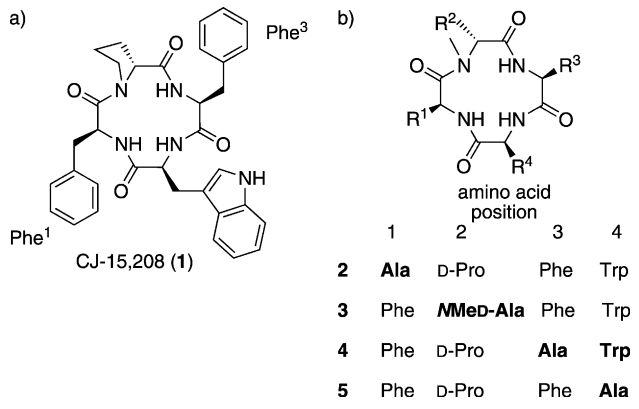


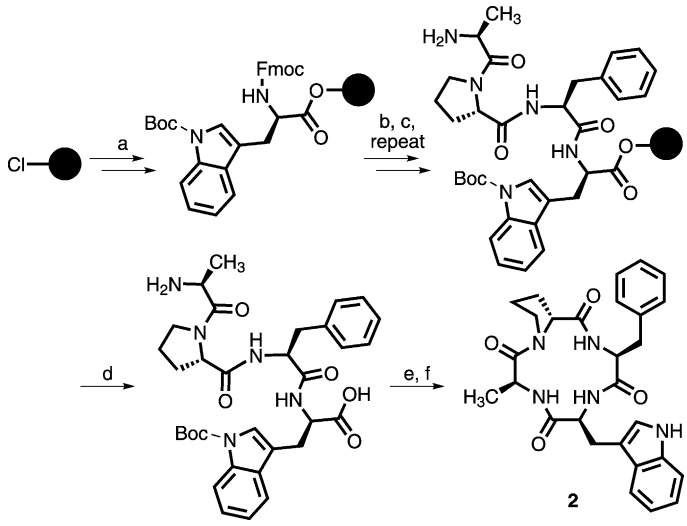
Figure 1. Structures of a) the cyclic tetrapeptide CJ-15,208 (**1**) and b) alanine analogues **2**–**5**. The residues are numbered 1–4, arbitrarily starting with the Phe C-terminal to the Trp residue.

the pharmacological activity observed in vivo. Herein we report the results of these initial studies, including both basic in vitro and in vivo characterization of these analogues of CJ-15,208.

Results and Discussion

Synthesis

The cyclic tetrapeptides were synthesized by a combination of solid-phase synthesis of the linear tetrapeptide precursors, followed by cyclization in solution (the synthesis of *cyclo*[Ala-D-Pro-Phe-Trp] (**2**) is shown in Scheme 1) using the optimized procedure described for CJ-15,208.[11] The cyclizations were performed by slow addition of the linear peptide precursor to the coupling reagent HATU and DIEA in DMF; under these conditions, the formation of the dimeric cyclic octapeptide is minimal.[11] The peptides were purified by reversed-phase HPLC.



Scheme 1. Synthesis of peptide **2**. *Reagents and conditions*: a) Fmoc-AA, DIEA, CH_2Cl_2/DMF (4:1), 6 h, RT; b) piperidine/DMF (1:4), 2×20 min, RT; c) Fmoc-AA, PyBOP, HOBt, DIEA, CH_2Cl_2/DMF (1:1), 2–4 h, RT; d) 1% TFA in CH_2Cl_2, 10×2 min, RT; e) HATU, DIEA, DMF, 12 h addition + 12–24 h reaction, RT; f) TFA/CH_2Cl_2 (1:1), 30 min, RT. Black spheres: 2-chlorotrityl chloride resin.

In vitro pharmacological characterization

The peptides were evaluated for opioid receptor affinity in radioligand binding assays using cloned opioid receptors.[13] Substitution of alanine had a large effect on affinities for KOR with generally less effect on affinities for MOR (Table 1). Unexpectedly, substitution of Phe[1] (see Figure 1 for notation) increased both KOR and MOR affinities by 4.4- and 19-fold, respectively. In contrast, substitution of the other three residues in the cyclic tetrapeptide with alanine decreased KOR affinity from 3- to 44-fold (Table 1), with the largest decrease occurring if the Trp residue is replaced by Ala, followed by substitution of Phe[3] (see Figure 1). These results suggest that Trp and Phe[3] are important for KOR affinity. These substitutions, however, did not decrease MOR affinity, but in one case (replacement of D-Pro by *N*Me-D-Ala in **3**) increased MOR affinity 4.4-fold, resulting in these three analogues exhibiting negligible selectivity for KOR over MOR. All of the peptides exhibited very low affinity for δ opioid receptors (DOR) in the binding assays.

Table 1. Opioid receptor affinities of alanine analogues of CJ-15,208.

Peptide	K_i [nM] ± SEM KOR	MOR	DOR	Selectivity KOR/MOR/DOR
2	8.03 ± 1.67	32.1 ± 3.9	8680 ± 1270	1/4.0/1080
3	113 ± 23	140 ± 9	1370 ± 70	1/1.2/12
4	663 ± 220	533 ± 28	> 10000	1.2/1/> 15
5	1550 ± 290	687 ± 81	> 10000	2.3/1/> 14
1[a]	35.4 ± 3.6	619 ± 87	4150 ± 3020	1/17.5/117

[a] Data from reference [12a].

The ligand-stimulated [^{35}S]GTPγS binding assay was used to assess the efficacy and potency of the cyclic tetrapeptides. None of the peptides exhibited appreciable stimulation of [^{35}S]GTPγS binding via either KOR or MOR, consistent with the lack of agonist activity of the parent peptide **1** in this assay.[12] The Ala analogue **2** is a reasonably potent antagonist of both dynorphin A-(1–13)NH_2 at KOR (K_B = 2.6 ± 0.8 nM) and [D-Ala[2],*N*MePhe[4],glycol]enkephalin (DAMGO) at MOR (K_B = 7.3 ± 1.6 nM), consistent with its affinities for KOR and MOR. Notably, it is 25-fold more potent as a KOR antagonist than the parent peptide **1** (K_B = 65.2 ± 1.6 nM), while retaining MOR antagonist potency similar to **1** (K_B = 10.2 ± 1.7 nM).

In vivo pharmacological characterization

The opioid activity of the cyclic tetrapeptides was determined in vivo by using C57Bl/6J mice in the 55 °C warm-water tail-withdrawal assay following intracerebroventricular (i.c.v.) administration. This initial evaluation was done following central administration to measure the inherent pharmacological activi-

ty of the analogues in vivo without the complications associated with distribution (i.e., blood–brain barrier penetration) that could affect activity following systemic administration. Like CJ-15,208, each of the alanine analogues exhibited antinociceptive activity in vivo, albeit with varying potencies (Figure 2).

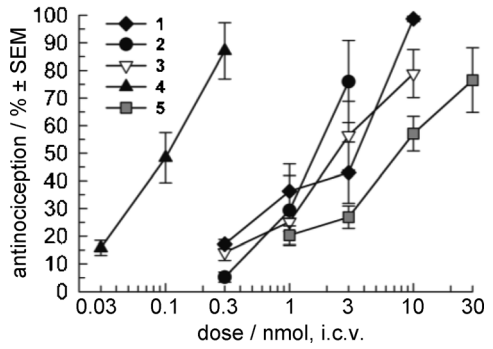


Figure 2. The antinociceptive activity of the cyclic tetrapeptides was assessed in vivo following i.c.v. administration in the 55 °C warm-water tail-withdrawal assay in C57Bl/6J mice. All points represent antinociception at peak response, which was 20 min (for **4**), 30 min (for peptides **1**, **2**, and **5**) or 40 min (peptide **3**). All points represent average percent antinociception ± SEM from 7–8 mice. Data for **1** from reference [12a].

Analogues **2** and **3** exhibited similar antinociceptive potencies to CJ-15,208 in this assay, with ED_{50} (and 95% confidence interval) values of 1.49 (0.39–7.41) and 2.43 (0.71–8.85) nmol for **2** and **3**, respectively, versus 1.74 (0.62–4.82) nmol for CJ-15,208. Interestingly, analogue **4** (ED_{50} = 0.10 (0.03–0.35) nmol) is 17-fold more potent than the parent peptide. Peptide **5**, in which the Trp residue is substituted by Ala, proved to be the least potent analogue, with an ED_{50} value of 6.97 (1.02–47.4) nmol, fourfold lower than the parent peptide.

While consistent with the in vivo activity of the parent peptide CJ-15,208, the antinociceptive activity of these analogues was surprising, given their lack of agonist activity in the [^{35}S]GTPγS assay in vitro and the relatively low opioid receptor affinities of analogues **3**–**5**. Therefore we evaluated whether the antinociceptive activity is mediated through opioid receptors. In our initial testing, the antinociceptive activity of each peptide was completely blocked by pretreatment with the nonselective opioid antagonist naloxone (Figure 3), verifying opioid receptor involvement. We subsequently examined which opioid receptors are involved in the antinociceptive activity by pretreating test subjects with antagonists selective for MOR, KOR, and DOR. The antinociceptive activity of each of the alanine analogues **2**–**5**, administered at a dose producing 50–80% antinociception, was almost completely blocked by pretreatment with the MOR-selective irreversible antagonist β-funaltrexamine (β-FNA, Figure 4). Of interest, pretreatment with the KOR-selective antagonist nor-BNI produced differing effects, significantly antagonizing the antinociceptive activity of only peptide **5**, with no effect on **3** or **4** (Figure 4). Pretreatment with nor-BNI decreased the antinociceptive activity of analogue **2** by >30%, but the difference in the presence versus absence of nor-BNI did not reach statistical significance. The

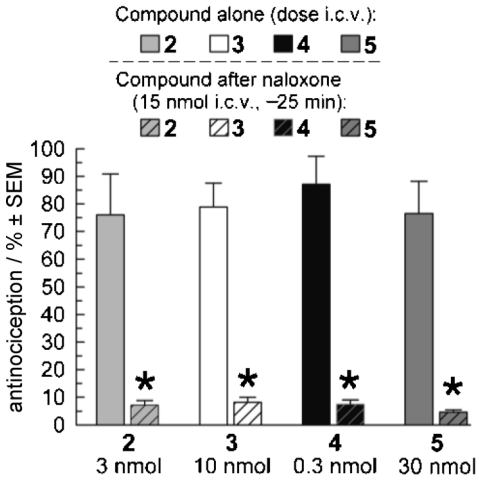


Figure 3. Cyclic tetrapeptide-induced antinociception is opioid receptor mediated. Peak antinociceptive activity of peptides **2** (3 nmol), **3** (10 nmol), **4** (0.3 nmol), and **5** (30 nmol) was determined in the 55 °C warm-water tail-withdrawal assay after i.c.v. administration to C57Bl/6J mice (open bars). Naloxone pretreatment (15 nmol, i.c.v., striped bars) 25 min prior to peptide administration significantly antagonized the effect of each cyclic tetrapeptide. Tail-withdrawal latencies were measured 30 min after injection of the cyclic tetrapeptide. Data represent average percent antinociception ± SEM from 7–8 mice. *: Significantly different from response of matching administered compound alone ($p < 0.05$), one-way ANOVA followed by Tukey's HSD post-hoc test.

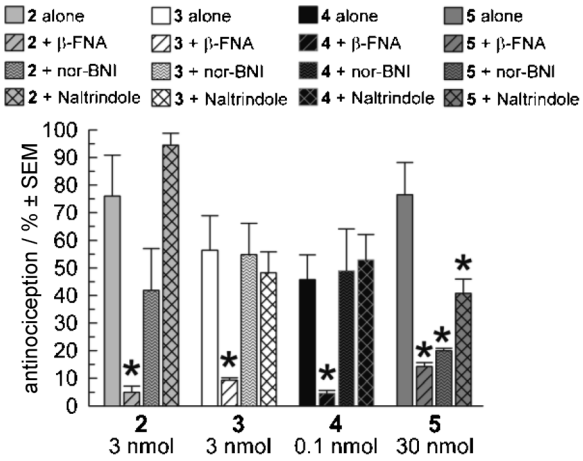


Figure 4. Opioid receptor-selective agonism by the cyclic tetrapeptides. The antinociceptive activity of peptides **2** (3 nmol), **3** (3 nmol), **4** (0.1 nmol), and **5** (30 nmol) was determined in the 55 °C warm-water tail-withdrawal assay after i.c.v. administration to C57Bl/6J mice (solid bars). Antinociception was also assessed 24 h after administration in mice pretreated with β-FNA (5 mg kg^{-1}, s.c.; diagonally striped bars) or nor-BNI (10 mg kg^{-1}, i.p.; wave-filled bars). Additional mice were pretreated with naltrindole (20 mg kg^{-1}, i.p., −15 min; hatched bars) before administration of one of the cyclic tetrapeptides. Tail-withdrawal latencies were measured in the mouse 55 °C warm-water tail-withdrawal test 30 min after injection of the cyclic tetrapeptides **2**, **4**, and **5**, or 40 min after peptide **3**. Data represent average percent antinociception ± SEM from 8–16 mice. *: Significantly different from response of matching administered compound alone ($p < 0.05$), one-way ANOVA followed by Tukey's HSD post-hoc test.

DOR-selective antagonist naltrindole significantly decreased the antinociceptive activity of only peptide **5**. Together, these results suggest that the antinociception induced by peptides **2**–**4** is mediated almost exclusively by MOR, although it is pos-

sible that KOR contributes to the antinociceptive activity of **2**. In contrast, all three receptors appear to contribute to the antinociceptive activity of **5**. These results are in contrast to the antinociceptive activity of **1**, which appears to be predominantly mediated by KOR, with a lesser contribution by MOR.[12a]

We next examined the peptides for antagonist activity following dissipation of the agonist activity. For the parent peptide **1** significant agonist activity was detected for up to 100 min after administration of the highest dose (10 nmol), whereas for analogues **2**–**5** significant agonist activity was detected for 70–80 min. To ensure that there was no residual agonist activity, the peptides were evaluated for antagonist activity 3 h after pretreatment. All of the analogues dose-dependently antagonized the antinociceptive effects of the KOR-selective agonist U50,488 (Figure 5). Peptides **2** and **4** appeared to

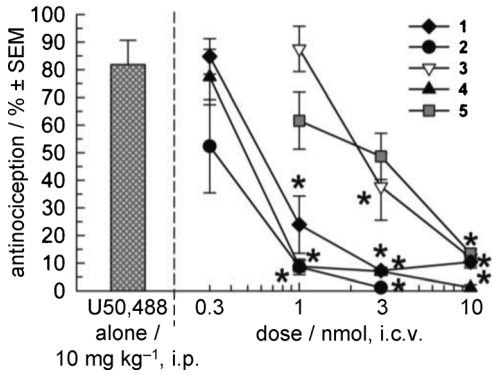


Figure 5. Dose-dependent antagonism of U50,488-induced antinociception by tested cyclic tetrapeptides. The antinociceptive effects of U50,488 (10 mg kg^{-1}, i.p.; thatched bar) were determined 40 min after administration in mice pretreated 3 h with peptides **1**–**5** in the 55 °C warm-water tail-withdrawal assay after i.c.v. administration. Data represent average percent antinociception ± SEM from eight mice. *: Significantly different from response of U50,488 ($p < 0.05$), one-way ANOVA followed by Tukey's HSD post-hoc test. Data for **1** from reference [12a].

be somewhat more potent than the parent peptide **1**, whereas peptides **3** and **5** are less potent as KOR antagonists than **1**. Importantly, the duration of the KOR antagonist activity for each of the peptides was relatively brief (< 18 h, Figure 6), substantially shorter than the duration of activity of the parent peptide **1**, which exhibits significant KOR antagonist activity for at least 24 h.[12a] The reason for the shorter duration of antagonist activity of the analogues relative to the parent peptide is unclear, but could be due to differences in hydrophobicity, with the more hydrophobic parent peptide being retained longer in the tissue.

The selectivity of the antagonist activity was next evaluated by examining the ability of the peptides to antagonize the antinociception produced by the MOR-preferring agonist morphine or the DOR-selective agonist SNC-80 (Figure 7). None of the peptides antagonized morphine. While peptide **2** did not significantly antagonize SNC-80, surprisingly, peptides **3** and **5** did antagonize this agonist (the decrease in the antinociception of SNC-80 by pretreatment with **4** was not significant).

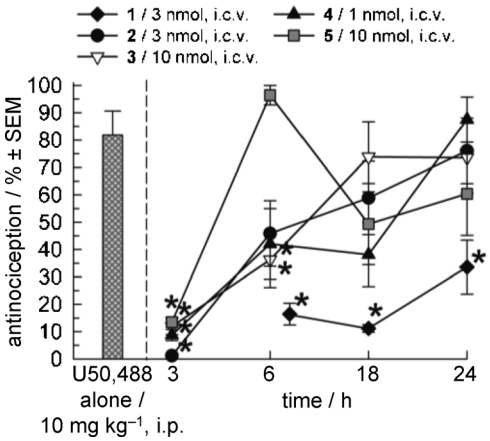


Figure 6. Duration of cyclic tetrapeptide-mediated antagonism of U50,488-induced antinociception in the mouse 55 °C warm-water tail-withdrawal test. Antinociception of U50,488 (10 mg kg^{-1}, i.p.; thatched bar) was determined in mice pretreated 3, 6, 18, or 24 h with peptides **2**–**5**, and for peptide **1** at 8, 18, and 24 h.[12a] Tail-withdrawal latencies were determined 40 min after agonist administration. Data represent average percent antinociception ± SEM from eight mice per point. *: Significantly different from response of U50,488 ($p < 0.05$), one-way ANOVA followed by Tukey's HSD post-hoc test.

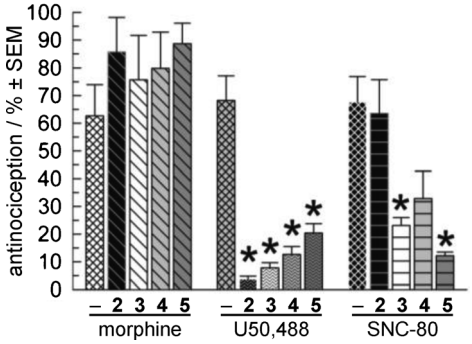


Figure 7. Receptor selectivity of antagonism by cyclic tetrapeptides in the mouse 55 °C warm-water tail-withdrawal test. Antinociceptive activity of morphine (10 mg kg^{-1}, i.p.; left group) was not decreased by a 3 h pretreatment of the mice with the indicated cyclic tetrapeptides **2** (10 nmol), **3** (1 nmol), **4** (1 nmol), or **5** (10 nmol). However, the antinociceptive effect of U50,488 (10 mg kg^{-1}, i.p.; center group) was significantly antagonized by pretreatment of the mice with any of the cyclic tetrapeptides. (For this set of tests peptide **2** was administered at 3 nmol, i.c.v.). In contrast, the antinociceptive effect of SNC-80 (100 nmol, i.c.v., right group) was significantly prevented only by pretreatment with peptides **3** and **5**. Tail-withdrawal latencies were measured in the mouse 55 °C warm-water tail-withdrawal test 40 min after injection of the known selective agonists. Data represent average percent antinociception ± SEM from 8–16 mice. *: Significantly different from response of matching administered agonist alone ($p < 0.05$), one-way ANOVA followed by Tukey's HSD post-hoc test.

This DOR antagonist activity was unexpected given the low affinity of these cyclic tetrapeptides for DOR.

Conclusions

The alanine analogues of CJ-15,208 exhibited in vivo pharmacological profiles that were unexpected based on their opioid receptor affinities and lack of agonist activity in the [^{35}S]GTPγS assay in vitro. All of the analogues exhibited antinociceptive

activity in the 55 °C warm-water tail-withdrawal assay, with analogue **4** exhibiting particularly potent agonist activity. This antinociceptive activity involves opioid receptors, as it is blocked by the nonselective opioid antagonist naloxone. Further examination with selective antagonists suggests that the antinociceptive activity of the more potent analogues **2**–**4** is predominantly if not entirely mediated through MOR activation, which contrasts with the antinociceptive activity of the parent peptide **1**,[12a] which appears to be predominantly mediated through activity at KOR with a smaller contribution by MOR. These alanine analogues also exhibited antagonist activity at KOR after dissipation of the agonist activity, which was unexpected, especially in the case of the potent antagonist **4**, given its low KOR affinity. The DOR antagonist activity of at least two of the analogues was also unexpected, given the very low DOR affinities of these compounds. Clearly, these analogues have different opioid activity profiles in vivo from what was expected from the in vitro assays and also from the results for the parent peptide **1**.

The in vivo data suggest that there are different structural requirements for agonist versus antagonist activity mediated by KOR and for the activation of KOR versus MOR. All three of the aromatic residues appear to contribute to agonist activity mediated by KOR. Interestingly, only the antinociceptive activity of the lower-potency agonist **5**, in which the Trp residue is replaced by Ala, was significantly antagonized by nor-BNI. All of the peptides, however, antagonized KOR in vivo, although peptide **5** also exhibited relatively low potency as an antagonist. All of the analogues exhibited agonist activity that was mediated predominantly by MOR, suggesting that only two of the aromatic residues are sufficient for activation of MOR. The analogues in which one of the Phe residues is replaced with alanine exhibited high potency both as MOR agonists and as KOR antagonists in vivo. These analogues are undergoing additional evaluation in vivo as lead compounds for potential development as analgesics.

While these peptides produce antinociception and antagonist activity that is clearly mediated through opioid receptors, the marked differences between the activity profiles in the in vitro versus in vivo assays suggest that these compounds elicit their opioid activity through more complex mechanisms than typical opioid receptor ligands. Notably, similar differences have been found between in vitro and in vivo opioid activity for other novel peptide-based antinociceptive compounds that are structurally distinct from these peptides.[14] Involvement of additional mechanisms in analgesic activity has been reported for other opioid peptides. For example, the indirect activation of opioid receptors by release of endogenous opioid peptides has been reported for several opioid peptides,[15] most notably for the potent and selective MOR peptides Dmt-DALDA (Dmt-D-Arg-Phe-Lys-NH_2, Dmt = 2′,6′-dimethyltyrosine) and endomorphin-2. A non-opioid mechanism (inhibition of norepinephrine uptake) was also reported to contribute to the antinociceptive effects of Dmt-DALDA.[16] Additional studies are being conducted to explore possible mechanisms for the observed antinociceptive activity of these cyclic tetrapeptides.

In conclusion, these unusual ligands represent valuable compounds for further study and as novel lead compounds, particularly analogues **2** and **4**, for the development of peptide-based opioid analgesics. These studies are ongoing in our laboratories.

Experimental Section

Materials: Reagents for peptide synthesis were obtained from the following sources: Fmoc (fluorenylmethoxycarbonyl)-protected amino acids (Novabiochem (EMD), San Diego, CA, USA), 2-chlorotrityl chloride resin (1.4 mmol g^{-1}, Novabiochem), coupling reagents HATU (2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Novabiochem), PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, Novabiochem) and HOBt (1-hydroxybenzotriazole, Fluka, Milwaukee, WI, USA), DIEA (*N*,*N*-diisopropylethylamine, Fluka), TFA (trifluoroacetic acid, Pierce, Rockford, IL, USA), and HPLC-grade solvents (Fisher Scientific, Pittsburg, PA, USA). The other solvents and routine chemicals were obtained from Fisher Scientific. HPLC analyses and purifications were performed on Vydac 218TP C_{18} reversed-phase columns (Grace Davison, 4.6×50 mm, 5 μm, and 22×250 mm, 10 μm, respectively).

Peptide synthesis:[11]

Solid-phase synthesis of linear peptide precursors. The linear peptide precursors were synthesized using Fmoc-protected amino acids on a 2-chlorotrityl chloride resin and a custom-made manual peptide synthesizer (CHOIR)[17] constructed in house. Following swelling of the resin in CH_2Cl_2 (2×10 min), the C-terminal Fmoc-protected amino acid (2 equiv) and (DIEA, 5 equiv) in CH_2Cl_2/*N*,*N*-dimethylformamide (DMF) (4:1, 5 mL per 0.5 g resin) were added to the resin, and the reaction gently agitated with N_2 gas for 6 h. Additional CH_2Cl_2 was added every 30 min to maintain the solvent volume, and additional DIEA (5 equiv) was added to the reaction every 2 h. The resin was washed with CH_2Cl_2/DMF (1:1, 5×), and quantitative Fmoc analysis[18] was then used to determine loading efficiency. A capping step was then performed using 15% MeOH and 5% DIEA in CH_2Cl_2 (2×10 min), and the resin was washed with CH_2Cl_2/DMF (1:1, 5×). The Fmoc group was then removed with 20% piperidine in DMF (2×20 min), and the resin was washed with CH_2Cl_2/DMF (1:1, 5×) and CH_2Cl_2 (5×).

Fmoc-protected amino acids (4 equiv) were coupled to the resin using PyBOP (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) in CH_2Cl_2/DMF (1:1) for 2–4 h. The resin was washed after the coupling reactions with CH_2Cl_2/DMF (1:1, 5×) and CH_2Cl_2 (5×). The reactions were monitored to determine completion using the Kaiser test for primary amines or the chloranil test for the secondary amine of Pro. The Fmoc group was then removed as described above, and the deprotection/coupling cycle repeated to assemble the linear tetrapeptides. Finally, the resin was washed with CH_2Cl_2/DMF (1:1, 5×), CH_2Cl_2 (10×), *i*PrOH (2×), hexane (2×), CH_2Cl_2 (2×), MeOH (2×), and finally CH_2Cl_2 (2×).

The peptides were cleaved from the resin using 1% TFA in CH_2Cl_2. Following swelling of the resin in CH_2Cl_2, the TFA solution was mixed with the resin (5 mL × 2 min × 10), and the cleavage solution was drained into a round-bottom flask. This procedure was repeated until all of the cleavage solution was collected in the flask. Following cleavage, the resin was washed with CH_2Cl_2 (2×) and MeOH (2×). The combined solutions were evaporated to give the

crude linear tetrapeptides, which were used in the cyclizations without purification.

Cyclization reaction and final deprotection. The linear peptides were cyclized as follows: The crude linear peptide (0.5 equiv) in DMF (5–10 mL) was added dropwise at a rate of 1.6 mL h^{-1} (using a KD Scientific single infusion syringe pump and a 10 mL syringe) to a solution of HATU (0.75 equiv, 1 mM) and DIEA (8 equiv) in DMF over 6 h. After 6 h a second portion of HATU (0.75 equiv) was added to the reaction in one portion, and a second portion of linear peptide (0.5 mmol) in DMF (5–10 mL) was added dropwise at a rate of 1.6 mL h^{-1} as described above. The reaction was then allowed to stir for an additional 12–24 h. Following removal of the solvent under reduced pressure, the residue was dissolved in EtOAc/Et_2O (4:1) or CH_2Cl_2 and the solution washed with 1 N citric acid (2×), saturated bicarbonate (2×) and brine (2×). The organic layer was separated, dried (Na_2SO_4), and the solvent was removed under reduced pressure to give the crude cyclic peptide. This workup was not performed for peptide **5** because of its water solubility; instead, following removal of the DMF the crude peptide was dissolved in water and lyophilized.

The Boc (*tert*-butyloxycarbonyl) group on the indole group of Trp in the cyclic precursors of **2**–**4** was then removed by treating a solution of the cyclic peptide in CH_2Cl_2 (1 mL) with 50% TFA in CH_2Cl_2 (2 mL) for 30 min. The solution was then evaporated, and the peptide triturated with 10% aqueous AcOH; the peptide was then dried by lyophilization.

Purification and characterization. The cyclic peptides were purified by reversed-phase HPLC (30–70% aqueous MeOH over 40 min, except for peptide **5**, for which the gradient was 20–60% aqueous MeOH over 40 min). The cyclic peptides were characterized by reversed-phase HPLC and electrospray ionization mass spectrometry (see Supporting Information).

In vitro pharmacological evaluation

Radioligand binding assays. Opioid receptor affinities were determined in radioligand binding assays using membranes from Chinese hamster ovary (CHO) cells stably expressing KOR, MOR, or DOR as previously described.[13] Incubations with isolated membrane protein were performed in triplicate with 12 different concentrations from 0.1 nM to 10 μM of the cyclic tetrapeptides for 90 min in 50 mM Tris, pH 7.4, at 22 °C using [^{3}H]diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE as the respective radioligands for KOR, MOR, and DOR. Nonspecific binding was determined in the presence of 10 μM unlabeled dynorphin A-(1–13)NH_2, DAMGO, and DPDPE for KOR, MOR, and DOR, respectively. Reactions were terminated by rapid filtration over Whatman GF/B fiber filters using a Brandel M24-R cell harvester, and the filters were counted in 4 mL Cytocint (ICN Radiochemicals) using a Beckman LS6800 scintillation counter. IC_{50} values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using Prism software (GraphPad Software, La Jolla, CA, USA). K_i values were calculated from the IC_{50} values by the Cheng and Prusoff equation[19] using K_D values of 0.45, 0.49, and 1.76 nM for [^{3}H]diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE, respectively. The results presented are the mean ± SEM from at least three separate assays.

GTPγS assays. The binding of the GTP analogue [^{35}S]GTPγS to membranes was assayed following the method described by Siebenallar and Murray.[20] Binding was determined in a volume of 500 μL. The assay mixture contained 50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM magnesium acetate, 1 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 1 mg mL^{-1} bovine serum albumin, and ~100000 disintegrations per min (dpm) [^{35}S]GTPγS (0.1–0.2 nM). Approximately 10 μg KOR- or MOR-expressing CHO cell membrane protein was used per tube. Following 90 min incubation at 22 °C, the assay was terminated by filtration under vacuum on a Brandel (Gaithersburg, MD, USA) model M-48R cell harvester using Schleicher and Schuell Inc. (Keene, NH, USA) number 32 glass fiber filters. The filters were rinsed with 4×4 mL washes of ice-cold 50 mM Tris-HCl, pH 7.4, 5 mM MgCl, at 5 °C to remove unbound [^{35}S]GTPγS. Filter disks were then placed into counting vials to which 8 mL Biocount scintillation fluid (Research Products International Corp., Mount Prospect, IL, USA) was added. Filter-bound radioactivity was determined by liquid scintillation spectrometry (Beckman Instruments, Fullerton, CA, USA) following overnight extraction at room temperature. The amount of radioligand bound was <10% of the total added in all experiments. Specific binding is defined as total binding minus that occurring in the presence of 3 μM unlabeled GTPγS. Nonspecific binding was ~1% of the total binding at 0.1 nM [^{35}S]GTPγS.

To evaluate the peptides for agonist activity, the membranes were incubated with ten different concentrations of peptide (0.01 nM to 1 μM). The antagonist activity of the peptides was determined by measuring the EC_{50} of an agonist (dynorphin A-(1–13)NH_2 for KOR and DAMGO for MOR) in the absence or presence of four different concentrations (10 nM to 3 μM) of the peptide. The pA_2 was determined by Schild analysis,[21] and the results are reported as K_B values.

In vivo pharmacological evaluation

Animals. 317 adult male C57Bl/6J mice weighing 20–25 g were obtained from Jackson Labs (Bar Harbor, ME, USA), and were housed and cared for in accordance with the 2002 National Institutes of Health Guide for the Care and Use of Laboratory Animals and as approved by the Torrey Pines Institute for Molecular Studies Institutional Animal Care Committee, operating under the OLAW approval number A4618-01. All mice were group housed, four to a cage, in self-standing plastic cages within the animal care facility. The colony room was illuminated on a 12 h light–dark cycle, with the lights on at 7:00 each morning. Food pellets and distilled water were available ad libitum. Note that C57Bl/6J mice were selected for this study because of their established responses to thermal noxious stimuli and antinociceptive testing.[22] All compounds other than the peptides were obtained from Sigma (St. Louis, MO, USA).

Intracerebroventricular administration technique. Intracerebroventricular (i.c.v.) injections were made directly into the lateral ventricle according to the modified method of Haley and McCormick.[23] The volume of all i.c.v. injections was 5 μL, using a 10 μL Hamilton microliter syringe. The mouse was lightly anesthetized with isoflurane, an incision was made in the scalp, and the injection was made 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm.

Antinociceptive testing. The 55 °C warm-water tail-withdrawal assay was performed in C57Bl/6J mice as previously described.[24] Briefly, warm (55 °C) water in a 2 L heated water bath was used as the thermal nociceptive stimulus, with the latency of the mouse to withdraw its tail from the water taken as the endpoint. After determining baseline tail-withdrawal latencies, mice were administered a graded dose of compound though the i.c.v. route. Intracerebroventricular injections (5 μL, using a 10 μL Hamilton syringe) were performed as described above; the cyclic tetrapeptides were ad-

ministered in 50% DMSO/50% sterile saline (0.9%). To determine agonist activity, the tail-withdrawal latency was determined every 10 min following administration of a cyclic tetrapeptide for 3 h, or until latencies returned to baseline values.

A cutoff time of 15 s was used in this study; if the mouse failed to display a tail-withdrawal response during that time, the tail was removed from the water, and the animal was assigned a maximal antinociceptive score of 100%. At each time point, antinociception was calculated according to the following formula: percent antinociception = 100 × (test latency − control latency)/(15 − control latency).

To determine the opioid receptor selectivity of the agonist activity of peptides **2**–**5**, mice were pretreated with a single dose of β-FNA (5 mg kg^{-1}, s.c.) or nor-BNI (10 mg kg^{-1}, i.p.) 23.3 h in advance of administration of a graded dose of a cyclic tetrapeptide compound (0.1–30 nmol, i.c.v.). Additional mice were pretreated prior to the administration of a cyclic tetrapeptide compound with the nonselective opioid receptor antagonist naloxone (15 nmol, i.c.v., −25 min), or naltrindole (20 mg kg^{-1}, i.p., −15 min), with antinociceptive testing 40 min later. Reference agonists and antagonists were administered using sterile saline (0.9%) as the vehicle, except for SNC-80, which was dissolved in 35% DMSO/65% saline.

To determine antagonist activity, mice were pretreated with a cyclic tetrapeptide 150 min prior to administration of the MOR-preferring agonist morphine (10 mg kg^{-1}, i.p.), the KOR-selective agonist U50,488 (10 mg kg^{-1}, i.p.) or the DOR-selective agonist SNC-80 (100 nmol, i.c.v.). Antinociception produced by these established agonists was then measured 30 min after administration. Additionally, to determine the duration of KOR antagonist activity, additional mice were pretreated for 7.3, 17.3, or 23.3 h prior to administration of U50,488 as described above.

Statistical analysis. Radioligand binding results represent the mean ± SEM obtained from 3–5 independent experiments, each performed in triplicate. IC_{50} values were calculated by least-squares fit to a logarithm–probit analysis. The K_i values of unlabeled compounds were calculated from the equation $K_i = IC_{50}/(1+S)$, where S = (concentration of radioligand)/(K_D of radioligand),[19] and reported as the mean ± SEM of at least three independent experiments. K_B values from the GTPγS assay represent the mean ± SEM from 2–4 experiments.

All tail-withdrawal data points shown are the means of 7–16 mice, with SEM represented by error bars. Data for antinociception experiments were analyzed with ANOVA using the Prism 5.0 software package (GraphPad, La Jolla, CA, USA). Analyses examined the main effect of baseline and post-treatment tail-withdrawal latencies to determine statistical significance for all tail-withdrawal data. Significant effects were further analyzed using Tukey's HSD post-hoc testing. All data are presented as mean ± SEM, with significance set at $p < 0.05$.

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