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Nascent structure–activity relationship study of a diastereomeric series of kappa opioid receptor antagonists derived from CJ-15,208

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ABSTRACT

Cyclic tetrapeptide c[Phe-pro-Phe-trp] **2**, a diastereomer of CJ-15,208 (**1**), was identified as a potent dual κ/μ opioid receptor antagonist devoid of δ opioid receptor affinity against cloned human receptors: K_i (**2**) = 3.8 nM (κ), 30 nM (μ); IC_{50} ([^{35}S]GTPγS binding) = 140 nM (κ), 21 nM (μ). The D-tryptophan residue rendered **2** ca. eightfold and fourfold more potent at κ and μ, respectively, than the corresponding L-configured tryptophan in the natural product **1**. Phe analogs **3–10**, designed to probe the effect of substituents on receptor affinity and selectivity, possessed K_i values ranging from 14 to 220 nM against the κ opioid receptor with μ/κ ratios of 0.45–3.0. An alanine scan of **2** yielded c[Ala-pro-Phe-trp] **12**, an analog equipotent to **2**. Agents **2** and **12** were pure antagonists in vitro devoid of agonist activity. Ac-pro-Phe-trp-Phe-NH_2 **16** and Ac-Phe-trp-Phe-pro-NH_2 **17** two of the eight possible acyclic peptides derived from **1** and **2**, were selective, modestly potent μ ligands: K_i (**16**) = 340 nM (μ); K_i (**17**) = 360 nM (μ).

Mu, kappa and delta opioid receptors together with their endogenous ligands directly or indirectly regulate a remarkable spectrum of pharmacology in humans.[1] These include nociception, feeding behavior, stress, sleep, learning and memory, sexual activity, developmental and endocrine function, mental health and mood, locomotion, neurotransmitter release, gastrointestinal, renal and hepatic function, cardiovascular and immunological responses, respiration and thermoregulation. Of special note is the renaissance in opioid antagonists as pharmacological probes and human therapeutics. Naloxone (Narcan®) is a remedy for narcotic overdose. Naltrexone, a non-selective centrally-acting opioid antagonist, is marketed as a controlled release formulation (Vivitrol®) to treat alcoholism and substance abuse. In 2008, two peripherally-acting μ opioid antagonists received FDA approval in the United States: methylnaltrexone subcutaneous injection (Relistor®), an adjunct for reversing the constipative side effects associated with narcotic therapy in palliative care patients, and alvimopan (Entereg®) for the prevention and treatment of opioid-induced postoperative ileus. Targin®, launched in Germany in 2008, is a combination narcotic/opiate antagonist therapy providing analgesia while moderating constipation. The combination of hydrocodone (opioid agonist) with ultra-low dose naltrexone (Oxytrex®) is undergoing clinical investigation to provide pain relief with attenuated risk of tolerance and physical dependence. The clinical benefits of selective kappa and delta opioid receptor antagonists have yet to be defined. Animal data suggest their potential utility as antipsychotics, antitussives, immunosuppressants and drug abuse treatment.[2] In rodents, mixed μ agonists/δ antagonists display analgesic action with reduced tolerance and dependence liability.[3] Our continued interest in opioid ligands[4] prompted an investigation into the report of CJ-15,208, a natural product possessing kappa antagonist activity.

As part of a natural product screening program at Pfizer Japan to discover novel opioid receptor ligands, Saito and coworkers isolated cyclic peptide CJ-15,208 (**1**) from the fermentation broth of a fungus, *Ctenomyces serratus* ATCC15502 (Fig. 1).[5] The structure of CJ-15,208 was deduced by spectroscopic and chemical means to be the cyclic tetrapeptide c(Phe-pro-Phe-Xxx) where Xxx was a tryptophan residue of undefined stereochemistry. Using a guinea pig brain membrane preparation, the in vitro binding activity of **1** was assessed against the three opioid receptors. It was a modestly selective κ ligand: IC_{50} = 47 nM (κ), 260 nM (μ), 2600 nM (δ); μ/κ ratio = 5.5. The functional κ antagonist action of **1** was established upon reversal of the κ agonist asimadoline-suppressed twitch response in a rabbit vas deferens assay: EC_{50} = 1300 nM. Subsequently, Seale and coworkers at Glaxo Welcome established the

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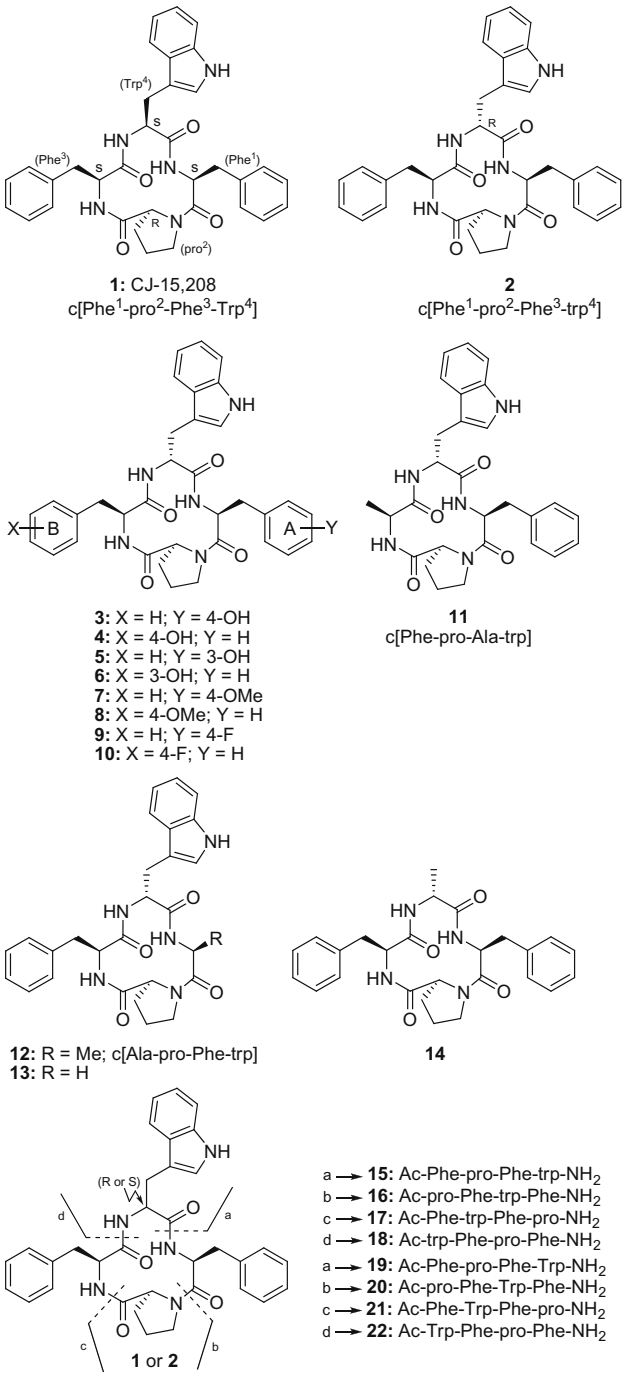

Figure 1. Cyclic and acyclic tetrapeptides.

L-configuration at Trp and hence, the absolute configuration of CJ-15,208 as c(Phe-pro-Phe-Trp) **1** by total synthesis.[6]

Cyclic peptide **1** was synthesized and evaluated against the cloned human μ, δ, and κ opioid receptors in our laboratories (Table 1).[7–9] The K_i values obtained were 29 nM (κ), 130 nM (μ) and 2000 nM (δ); μ/κ ratio = 4.5, comparable (within twofold) to the IC_{50} data reported for CJ-15,208 (Table 1).[4] The diastereomer of **1**, c[Phe-pro-Phe-trp] **2**, containing D-tryptophan was also synthesized. Surprisingly, **2** was nearly eightfold more active than **1** at κ and fourfold more active at μ and was devoid of δ receptor affinity: K_i for **2** = 3.8 nM (κ), 30 nM (μ) and >1000 nM (δ). Cyclic peptide **2** was more selective for κ (μ/κ ratio = 7.9) than **1**. Cyclic tetrapeptides **1** and **2** were robust functional antagonists at both κ and μ

Table 1
In vitro binding data for **1–22**

Number	K_i (nM)[a]				IC_{50} (nM)[d]	
	κ	μ	δ	μ/κ	κ	μ
1	29 (47)[b]	130 (260)[b]	2000 (2600)	4.5 (5.5)[b]	440	25
2	3.8	30	[c]	7.9	140	21
3	220	670	[c]	3.0	[e]	[e]
4	140	120	[c]	0.85	[e]	[e]
5	78	37	670	0.47	[e]	[e]
6	20	40	3400	2.0	[e]	[e]
7	66	170	[c]	2.6	[e]	[e]
8	14	43	[c]	3.1	[e]	[e]
9	28	210	[c]	7.5	[e]	[e]
10	21	48	[c]	2.3	[e]	[e]
11	1300	2200	[c]	1.7	[e]	[e]
12	5.2	22	[c]	4.2	5.0	48
13	270	770	[c]	2.8	[e]	[e]
14	[c]	[c]	[c]	—	[e]	[e]
15	[c]	[c]	[c]	—	[e]	[e]
16	[c]	340	[c]	—	[e]	[e]
17	[c]	360	[c]	—	[e]	[e]
18	[c]	[c]	[c]	—	[e]	[e]
19	[c]	[c]	[c]	—	[e]	[e]
20	[c]	[c]	[c]	—	[e]	[e]
21	[c]	[c]	[c]	—	[e]	[e]
22	[c]	[c]	[c]	—	[e]	[e]

[a] The binding affinities (K_i) of the peptides were determined by testing the ability of a range of concentrations of each peptide to inhibit the binding of the non-selective opioid antagonist, [^{3}H]diprenorphine, to cloned human μ, δ, and κ opioid receptors expressed in separate cell lines.[9] K_i values are the geometric means computed from at least three separate determinations.

[b] IC_{50} binding data for CJ-15,208 (Guinea pig brain membrane) taken from Ref. 5.

[c] K_i was >1000 nM.

[d] Antagonist potencies (IC_{50}) of the peptides were assessed by testing their ability to inhibit the stimulation of [^{35}S]GTPγS binding by EC_{80} concentrations of agonist (100 nM loperamide for μ and 50 nM U50,488H for κ) in membranes containing the cloned human μ and κ opioid receptors expressed in separate cell lines.[9] IC_{50} values are the geometric means computed from at least three separate determinations.

[e] Not determined.

as measured by their ability to inhibit the binding of [^{32}S]GTPγS mediated by the cloned human receptors. The IC_{50} values for **1** = 460 nM (κ), 25 nM (μ), while the IC_{50} value for the more potent **2** = 140 nM (κ), 21 nM (μ). In the functional assay, **2** was a more potent kappa antagonist than **1**.

Because c[Phe-pro-Phe-trp] **2** was the more active and selective opioid antagonist, it was targeted for analog synthesis. Of particular interest was the exchange of Phe residues for Tyr and *meta*-Tyr ((L-)-3-hydroxyphenylalanine). The Tyr residue is commonly found in opioid peptide ligands. Many nonpeptide opiate ligands contain a phenol. The phenolic moiety is a salient component of the unifying message-address concept of opioid ligand binding proposed by Portoghese.[10] It is well documented that modification of this aromatic hydroxyl group, for example, OH→OMe or H, leads to a striking loss of receptor affinity in both peptide and nonpeptide ligands. It was therefore hypothesized that Phe→Tyr in **2** may potentially yield analogs with enhanced opioid receptor binding affinity. The first pair of analogs prepared were c[Tyr-pro-Phe-trp] **3** and c[Phe-pro-Tyr-trp] **4**. Both of the peptides suffered a substantial loss in binding affinity at κ and μ. The loss in κ affinity was 58-fold in **3** and 37-fold in **4**. The corresponding *m*-Tyr analogs, c[*m*Tyr-Phe-pro-Phe-trp] **5** (K_i = 78 nM, (κ)) and c[Phe-pro-Phe-*m*Tyr -trp] **6** (K_i = 20 nM, (κ)) were also less active than **2**. However, the *meta* disposed hydroxyl group in **5/6** was significantly better tolerated than the *para* disposed hydroxyl group in **3/4**. Peptide **6** displayed a binding profile very similar to **1**, but with a threefold increase in μ affinity (μκ ratio = 2).

The hydrogen-bond donating phenol in **3/4** was converted to a hydrogen-bond acceptor by conversion to the methyl ether (Tyr→

Tyr(OMe)). The new peptides **7/8** showed improved binding at κ and μ relative to **3/4**. Peptide c[Tyr(OMe)-pro-Phe-trp] **7** had a K_i = 66 nM at κ. Peptide c[Phe-pro-Tyr(OMe)-trp] **8** possessed a K_i = 14 nM for κ and 43 nM for μ. Although **8** represents a 10-fold improvement in binding versus its Tyr congener **4**, it underperformed **2** by ca. fourfold, suggesting no benefit of the methoxy substituent as a H-bond acceptor. Peptide analogs **9** and **10** containing *p*-fluorophenylalanine (electron-withdrawing group, H-bond acceptor) did not improve binding affinity versus **2**, but rather provided ligands with κ affinities comparable to **1**: for **9** K_i = 28 nM (κ) and **10** K_i = 20 nM (κ). The μ affinity was much more sensitive to fluorine substitution in **9** K_i = 210 nM (μ) versus **10** K_i = 48 nM (μ). Looking across the peptide pairs **3/4**, **5/6**, **7/8** and **9/10**, in each case the [Phe^1] residue (phenyl ring A; analogs **4**, **6**, **8**, and **10**) was more tolerant of aromatic substitution than [Phe^3] (phenyl ring B; analogs **3**, **5**, **7**, and **9**).

At this juncture an alanine scan was performed by systematically replacing the tryptophan and phenylalanine residues with alanine. Analog **11** ([Phe^3]→[Ala^3]) was ca. 500-fold less active than **2** indicating the importance of the aromatic ring for activity. In contrast, analog **12** ([Phe^1]→[Ala^1]) was equipotent with **2**. The K_i for **12** = 5.2 nM (κ), 22 nM (μ), >1000 nM (δ) and displayed robust functional antagonist potency: IC_{50} = 5.0 nM (κ), 48 nM (μ). These results were consistent with the observation that [Phe^1] is less sensitive to aromatic substitution than [Phe^3].

A conformational search of **12** verified the relative rigidity of the 12-membered cyclic core (Fig. 2). In the cluster of lowest energy conformers, amide bonds alternate 180° with one another and the [Ala^1], [Phe^3] and [trp^4] side chains occupy pseudoequatorial positions on the ring. Removing the alanine side chain ([Ala^1]→[Gly^1]; **12**→**13**) resulted in ca. 50-fold and 35-fold decreased potency at κ and μ, respectively. A comparison of low energy conformation clusters of **12** and **13** reveal virtually complete overlap of the macrocyclic rings and no difference in disposition of the pro-Phe-trp side chains (data not shown). This suggests that the difference in potency between **12** and **13** may be due to a loss of a critical hydrophobic contact between the Ala methyl group and the receptor binding pocket. Analog **14** ([trp^4]→[ala^4]) was also inactive in the binding assay at the test concentration of 10,000 nM. Collectively, these observations are the hallmark of **12** acting as a reverse peptide turn mimetic whose biological activity is principally dependent upon the contacts of the cyclic peptide's side chains with the receptor and independent of backbone structure.[11]

In a final study, it was of interest to determine the contribution of the cyclic topography of **2** to receptor affinity. To this end, four acyclic peptides **15–18** were prepared. These were derived by systematic single amide bond cleavage (rupture points a–d) in **2**. In each new acyclic peptide, the N-terminus was capped with an acetyl group while the C-terminus was a primary amide. This was carried out so that **15–18** possessed no positive or negative charge, thereby mimicking the neutral, noncharged character of the cyclic peptide. Evaluation of **15–18** in the cloned human receptor binding assays revealed that two of the acyclic peptides, Ac-pro-Phe-trp-Phe-NH_2 **16** and Ac-Phe-trp-Phe-pro-NH_2 **17**, displayed selective, but modest potency at the μ receptor: K_i (**16**) = 340 nM (μ); K_i (**17**) = 360 nM (μ). The corresponding acyclic peptides **19–22** derived from **1** were all inactive. Clearly the cyclic nature of **2** plays an absolute role in aptly orienting the amino acid side chains for κ opioid receptor affinity. The motif Phe-trp-Phe present in acylic peptides **16** and **17** appears necessary to impart μ receptor affinity and selectivity. Interestingly, the diastereomeric motif Phe-Trp-Phe (**21** and **22**) was not recognized by the opiate receptors.[12]

In summary, c[Phe-pro-Phe-trp] **2**, a diastereomer of CJ-15,208, is a potent dual κ/μ opioid receptor antagonist devoid of δ opioid receptor affinity. The D-tryptophan residue rendered **2** ca. eightfold and fourfold more potent at κ and μ, respectively, than the corresponding L-configured tryptophan in the natural product **1**. Phe analogs **3–10** of **2** were designed to probe the effect of electron withdrawing/donating- and hydrogen bond donating/accepting substituents on receptor affinity and selectivity. The analogs possessed K_i values ranging from 14 to 220 nM against the κ opioid receptor with μ/κ ratios of 0.45–3.0. [Phe^3] proved more sensitive to substituent effects than [Phe^1]. Replacing [Phe^1] with Ala yielded c[Ala-pro-Phe-trp] **12**, an simplified analog whose affinity was equipotent to **2** and ca. 20-fold more potent as a functional antagonist at κ. In addition, acyclic peptides **16** and **17**, two of the eight possible acyclic peptides derived from **1** and **2** are selective, modestly potent μ ligands: K_i (**16**) = 340 nM (μ); K_i (**17**) = 360 nM (μ). These peptides share a common motif, Phe-trp-Phe, unique among opioid ligands.

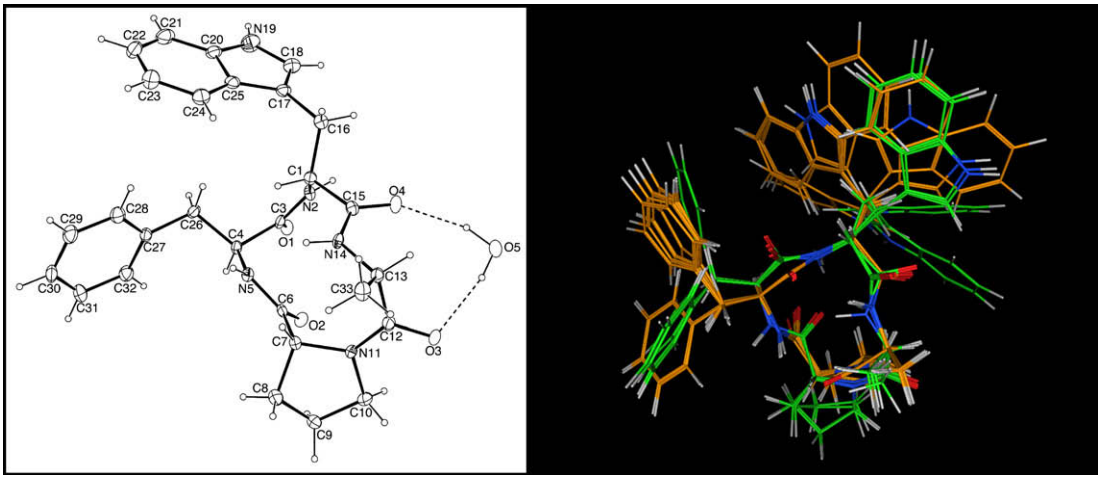

Figure 2. X-ray crystal structure and overlay of low energy conformations of c[Ala-pro-Phe-trp] **12**. The cluster of seven lowest energy conformers of the active cyclic peptide **12** is shown in orange. These conformers are all less than 5 kcal/mol over the lowest energy conformer found and share a common macrocyclic ring conformation where all the amide bonds are trans. The cluster of structures in green is 2.7–5 kcal/mol higher than the orange set and represent an alternative macrocyclic ring conformation where the amides between [trp^4] and [Ala^1] residues and between [Ala^1] and [pro^2] residues are inverted relative to the lowest energy conformer.[13] ORTEP diagram of **12** shows a water molecule hydrogen bonded to the pro and Ala carbonyl oxygen atoms and a cis amide bond between [Phe^3] and [trp^4] with trans amide bonds between the other amino acid residues. This conformation is ca. 16 kcal/mol higher in energy than the computed lowest energy conformation and may result from crystal packing forces.[14]

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13. Conformations of **12** and **13** were generated using the stochastic conformation search protocol and MMFF94x forcefield in MOE 2008.10 (Chemical Computing Group, Montreal, Quebec, Canada, www.chemcomp.com). Forcefield charges were assigned prior to conformation generation. A 20 kcal/mol energy cutoff was used to thoroughly sample conformational space, chiral inversion was not permitted, but rotation around amide bonds was allowed. The conformers were aligned by superposing the backbone atoms of the cyclic tetrapeptide prior to visual inspection of low energy structures.
14. Crystallographic data for compound **12** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 728552. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif Author P.J.C. determined the X-ray strucutre of **12**.