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Modifications of the cyclic mu receptor selective tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]NH_2 (Et): effects on opioid receptor binding and activation

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Abstract: The previously described cyclic mu opioid receptor-selective tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]NH_2 (Et) (JOM-6) was modified at residues 1 and 3 by substitution with various natural and synthetic amino acids, and/or by alteration of the cyclic system. Effects on mu and delta opioid receptor binding affinities, and on potencies and efficacies as measured by the [^{35}S]-GTPγS assay, were evaluated. Affinities at mu and delta receptors were not influenced dramatically by substitution of Tyr^1 with conformationally restricted phenolic amino acids. In the [^{35}S]-GTPγS assay, all of the peptides tested exhibited a maximal response comparable with that of fentanyl at the mu opioid receptor, and all showed high potency, in the range 0.4–9 nM. However, potency changes did not always correlate with affinity, suggesting that the conformation required for binding and the conformation required for activation of the opioid receptors are different. At the delta opioid receptor, none of the peptides were able to produce a response equivalent to that of the full delta agonist BW 373,U86 and only one had an EC_{50} value of less than 100 nM. Lastly, we have identified a peptide, D-Hat-c[D-Cys-Phe-D-Pen]NH_2 (Et), with high potency and $>$1000-fold functional selectivity for the mu over delta opioid receptor as measured by the [^{35}S]-GTPγS assay.

Abbreviations: Boc, *t*-butyloxycarbonyl (Boc); BW 373,U86 ($\pm$)-[1(S*),2α,5β]-4-[[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl]-*N*,*N*-diethyl-benzamide hydrochloride; DAMGO, [D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin; DCC, dicyclohexylcarbodiimide; Δ^EPhe, dehydro(E)phenylalanine; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; EC_{50}, dose required to exhibit 50% of the observed effect; EDTA, ethylenediaminetetraacetic acid; Et, dithioethane or

-S-CH_2-CH_2-S-; GDP, guanosine 5′-diphosphate; GTPγS, guanosine-5′-O-(3-thio)triphosphate; Hat, 6-hydroxy-2-aminotetralin-2-carboxylic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; HOBt, 1-hydroxybenzotriazole; Pen, penicillamine; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; *t*-Hpp, *trans*-3-(4′-hydroxyphenyl)proline; TLC, thin-layer chromatography; Tris, tris[hydroxymethyl]aminomethane; U69,593, 5α,7β,8γ-(-)-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide.

The existence of at least three types of opioid receptors, namely mu, delta and kappa, is widely accepted. Study of the interactions of structurally diverse ligands with the opioid receptors has led to the identification of pharmacophore models, which detail the spatial arrangement of structural features common to all ligands of a particular receptor. These models can be further refined through the design and study of conformationally restricted ligands, many of which display selectivity in binding to one or other opioid receptor.

The endogenous opioid pentapeptides lend themselves well to this approach. The cyclic disulfide-containing analog of Leu-enkephalin, [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), displays such high affinity and selectivity for the delta opioid receptor that it is considered the standard delta ligand (1). We have previously described a series of conformationally restricted disulfide- or dithioether-containing tetrapeptides that can be thought of as des-Gly^3 analogs of DPDPE (2), with the general structure depicted in Fig. 1. When cyclization is via a disulfide bond (i.e. $n = 0$, ring size 11 atoms) and the C-terminus is carboxyl, peptides show selectivity for the delta receptor, with affinity dependent upon the amino acid substitutions involved. For example, H-Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13), shows higher affinity for the delta receptor than DPDPE, with similar selectivity over the mu receptor. When JOM-13 is modified to include a C-terminus carboxamide, giving H-Tyr-c[D-Cys-Phe-D-Pen]NH_2 (JOM-5), delta receptor affinity is diminished while mu receptor affinity is enhanced, such that JOM-5 actually shows moderate mu receptor selectivity (2). Increasing the ring size of JOM-5 from 11 to 13 atoms by use of the dithioethane linkage (Et; $n = 2$; Fig. 1) gives JOM-6, H-Tyr-c[D-Cys-Phe-D-Pen]NH_2 (Et). JOM-6 exhibits greatly increased mu receptor affinity compared with JOM-5 with high selectivity (80-fold) for the mu opioid receptor (2).

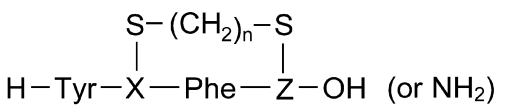

Figure 1. Structure of tetrapeptides, showing Tyr in position 1. X, D-Cys or D-Pen (Pen = penicillamine = β,β-dimethylcysteine). Z, D- or L-Cys or D- or L-Pen, $n = 0$ or 2.

Three of the key elements of pharmacophore that govern binding to both the mu and delta opioid receptor are contained within the Tyr^1 residue, namely the amino group, the phenolic aromatic and the hydroxyl group. The relative positions of the aromatic rings of the first and third residues are important both for binding and selectivity (3, 4). In addition, the aromatic ring of the Phe^3 residue, cyclic peptide ring size and C-terminal substitution also play important roles, particularly as determinants of mu/delta selectivity. As discussed above, smaller ring size and carboxylic acid C-terminal function favor delta binding, while a larger ring and carboxamide C-terminal function favor mu binding.

We have now prepared four analogs of JOM-6 which examine the effects of conformational restriction of Tyr^1 or Phe^3 and altered peptide ring size on the ability to bind to mu, delta and kappa receptors. In addition we have evaluated relative efficacy *in vitro* using the [^{35}S]-GTPγS assay as a functional measure (5). These alterations to the parent peptide reveal interesting differences in the relative affinities of this group of enkephalin- and dermorphin/deltorphin-like peptides at mu and delta opioid receptors. Also the results suggest that the structural requirements for binding to the mu or delta opioid receptor are not necessarily the same as the requirements for activation of the receptor.

Experimental Procedures

Peptide synthesis

All peptides (Table 1) were prepared using standard solid-phase methods similar to those described previously for the synthesis of JOM-6 (2), using chloromethylated polystyrene (Merrifield) resin cross-linked with 1% divinylbenzene. Trifluoroacetic acid (TFA) was used for deprotection and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were employed to facilitate coupling. Alpha-amino functions were *t*-butyloxycarbonyl (Boc) protected, and *p*-methylbenzene protection was employed for the labile side chain sulfhydyl groups of Cys and Pen. Simultaneous deprotection and cleavage from the resin were accomplished by treatment with anhydrous hydrogen fluoride in the presence of 5% *p*-cresol and 5% *p*-thiocresol for 45 min (6) at 0°C. Prior to cyclization, linear peptides were purified by reverse-phase high-performance chromatography (RP-

Table 1. **Physiochemical data for cyclic tetrapeptides**

Peptide structure Amino acid sequence	Bridge[a]	Analog	RP-HPLC[b] Elution time (min)	TLC R_f[c] A	B	FAB-MS [MH]+
L-*t*-Hpp-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**1**	30.8	0.67	0.78	614
L-Hat-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**2**	29.1	0.81	0.90	614
D-Hat-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**3**	29.5	0.82	0.93	614
Tyr-c[D-Cys-Δ^E-Phe-D-Pen]NH_2	S–S	**4**	27.6	0.48	0.57	558

a. Bridge between the second and fourth amino acids. S–Et–S denotes -S–CH_2–CH_2–S-. b. RP-HPLC as described in Experimental Procedures. c. TLC solvent systems described in Experimental Procedures.

HPLC) on a Vydac 218TP C-18 column (2.5 × 22 cm) using the solvent system 0.1% TFA in water/0.1% TFA in acetonitrile by a gradient of 10–50% organic component in 40 min. The disulfide-containing analog **4** was prepared by treatment of an aqueous solution (pH 8.5) of the corresponding linear free sulfhydryl-containing species with $K_3Fe(CN)_6$ (1). Cyclization to dithioether-containing analogs (**1**, **2** and **3**) was accomplished by treatment of a dilute solution of the linear free sulfhydryl-containing species in dimethyl formamide with potassium *tert*-butoxide followed by the addition of dibromoethane (7). All peptides were then purified by RP-HPLC as described above and pure fractions were lyophilized. Final product confirmation was obtained by fast-atom bombardment mass spectrometry (FAB-MS). In all cases the anticipated molecular masses were confirmed by FAB-MS. Final products were also analyzed by TLC using two solvent systems: solvent A, *n*-BuOH/HOAc/H_2O (4:1:5, v/v, organic phase only); and solvent B, *n*-BuOH/pyridine/HOAc/H_2O (15:10:3:12, v/v). The unusual amino acids *t*-Hpp and Hat were prepared as racemic mixtures as described previously (8, 9) and were used without resolution. The resulting diastereomeric peptide pairs were separated by HPLC as described above, and were individually subjected to binding assays. Stereochemistry for the diastereomeric Hat[1] analogs was determined by comparing HPLC elution times for the linear, free-sulfhydryl-containing form of these peptides with the retention times for the free sulfhydryl form of the unequivocally assigned, corresponding peptides with C-terminal carboxylate functions (9) under acidic (fully protonated) conditions. The L-*t*-Hpp-containing analog (**1**) was identified tentatively based on its 100-fold higher binding affinity compared with its diastereoisomer (data not shown), consistent with the well-established observation that L-stereochemistry at residue 1 is highly preferred at opioid binding sites. Molecular masses from FAB-MS, HPLC retention times and R_f values from TLC analysis are summarized in Table 1.

Membrane preparation for biological assays

Guinea-pig brain homogenates were used in the ligand-binding assays. Briefly, guinea-pig brains (Pel-Freez Biologicals) were suspended in cold 50 mM Tris buffer, pH 7.4 and homogenized using a Brinkman Polytron. The homogenate was centrifuged for 15 min at 14 000 *g* at 4°C and the supernatant discarded. The pellet was resuspended in cold 50 mM Tris buffer, pH 7.4, homogenized and recentrifuged. The pellet was suspended and the homogenate incubated at 37°C for 30 min to release endogenous opioids. After recentrifugation the pellet was resuspended at a final tissue concentration of ≈0.05 g/mL in cold 50 mM Tris buffer, pH 7.4 and stored in aliquots at –80°C.

For the [^{35}S]GTPγS binding assay, rat glioma cells stably transfected with either the rat mu opioid receptor ($C_6\mu$) (10) or the rat delta opioid receptor ($C_6\delta$) (11) (both kindly provided by Dr H. Akil) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and Geneticin at 1 mg/mL. Briefly, cells were grown to confluency in monolayers at 37°C in a humidified 5% CO_2 atmosphere. The cells were harvested by agitation in HEPES (20 mM, pH 7.4)-buffered saline containing 1 mM EDTA. After centrifugation at 500 *g*, the cell pellet was suspended in buffer (pH 7.4) containing 20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2.6H_2O$ (buffer A) and homogenized using a Tissue Tearor. The resultant homogenate was centrifuged at 40 000 *g* and the pellet collected, washed in buffer A and recentrifuged. The pellet was finally resuspended in buffer A to give a protein concentration of 1–2 mg/mL and stored in aliquots at –80°C. All procedures were performed at 4°C.

Radioligand-binding assays

Binding assays were based on the displacement by the test compounds of radiolabeled ([^{3}H]) ligands from opioid

receptors in guinea-pig brain membranes. The labeled ligands used were [D-Ala^2, NMePhe^3, Gly^5-ol]enkephalin (DAMGO) (mu ligand; 0.6 nM), DPDPE (delta ligand; 1.8 nM) and U69593 (kappa ligand; 0.9 nM) all from Amersham. The receptor binding assays were performed as described previously (12, 13). Briefly, the assay mixture, containing the membrane suspension in 50 mM Tris buffer (pH 7.4), radiolabeled ligand and test compound, was incubated at 25°C in triplicate for an appropriate amount of time to allow binding to reach equilibrium (either 75 or 90 min). Subsequently, the samples were filtered rapidly and the radioactivity on the filter paper determined by liquid scintillation counting. Inhibition of radiolabeled-ligand binding by the test compounds was computed from maximal specific binding, determined with an appropriate excess of unlabeled naloxone (10 μM). IC_{50} values were obtained by linear regression from plots relating the inhibition of specific binding to the log of 12 different ligand concentrations, using the computer program LIGAND (Biosoft Software) (14). K_i values were calculated using values for K_D of each radioligand determined previously from saturation binding assays (15).

[^{35}S]-GTPγS binding assay

Agonist stimulation of [^{35}S]-GTPγS binding in cell lines containing cloned receptors was measured as described previously (3). Briefly, membranes prepared from $C_6\mu$ or $C_6\delta$ cells as described above were incubated for 60 min at 30°C in buffer A containing [^{35}S]-GTPγS (100 pM), GDP (10 μM) and varying concentrations of ligand in a total volume of 1 mL. Basal binding of [^{35}S]-GTPγS was determined in the absence of unlabeled ligand, and maximal stimulation was defined using fentanyl (10 μM) in $C_6\mu$ cells or BW 373,U86 (10 μM) in $C_6\delta$ cells. Bound and free [^{35}S]-GTPγS were separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting. EC_{50} values were calculated using GraphPad Prism, version 2.01 (San Diego, CA).

Results and Discussion

The following modified and naturally occurring amino acids were incorporated as residue 1 substitutions in JOM-6, Tyr-c[D-Cys-Phe-D-Pen]NH_2 (Et); *trans*-hydroxyphenylproline (Hpp) (**1**), L-hydroxyaminotetralin (L-Hat) (**2**) and D-hydroxyaminotetralin (D-Hat) (**3**). Modification of the ethane bridge to a disulfide bond and replacement of the phenylalanine at position 3 with Δ^E-phenylalanine gave **4**, Tyr-c[D-Cys-Δ^EPhe-D-Pen]NH_2.

Radioligand-binding assays

Affinities of the cyclic peptides for mu, delta and kappa opioid receptors are shown in Table 2 and compared with values for DPDPE and DAMGO. The *trans*-hydroxyphenylproline (L-*t*-Hpp^1) derivative **1**, but not its diastereomer (data not shown), exhibited affinity for both mu and delta receptors equivalent to that of the parent compound JOM-6. The *trans*-hydroxyphenylproline residue restricts rotation about the Cα–Cβ but not the Cβ–Cγ bonds, thus reducing the conformational space accessible to the phenolic ring. However, the fact that **2** maintained the binding profile of JOM-6 despite Cα–Cβ rotational restriction implies that the required conformation(s) for binding to the opioid receptors lies within the limited accessible conformational space of the L-*t*-Hpp^1 residue. This has been seen previously for delta selective analogs of JOM-13 containing the L-*t*-Hpp^1 residue (8).

The hydroxyaminotetralin residue (Hat^1) restricts rotation about both the Cα–Cβ and Cβ–Cγ bonds through the formation of a bicyclic structure via cyclization to the α carbon, greatly restricting the number of possible conformers. The diastereomeric Hat^1 containing analogs **2** (L) and **3** (D) both had affinities at the mu opioid receptor identical to that of the parent peptide JOM-6. Delta selective D-Hat and L-Hat analogs of the peptide JOM-13 have previously been shown to exhibit similar binding affinities, which are reduced, but not abolished, when compared with the parent compound (9). As a consequence of its slightly reduced affinity for the delta receptor, **3** had improved selectivity for the mu opioid receptor compared with JOM-6, and at ≈ 150-fold it was the most selective of the peptides tested here.

The ability of both the D- and L-Hat^1 peptides to bind with high affinity to the mu, and indeed the delta, receptor is intriguing, since peptides **2** and **3** can never be configured in such a way as to allow superimposition of the phenolic rings and primary amine groups of the L- and D-Hat^1 residues, two of the essential pharmacophoric elements for binding to both the mu and delta receptors. However, the stereoisomeric amino acids can be configured such that both the primary amine and peptide amide groups, and hence the cyclic peptide system of both molecules, are superimposed. The cyclohexyl rings of both are also overlapped, although in slightly different conformations. There is a difference in the position of the tyramine phenyl rings, which are in the same

Table 2. **Opioid receptor binding profiles of cyclic tetrapeptides**

Peptide structure Amino acid sequence	Bridge[a]	Analog	Binding K_i (nM) mu	delta	kappa	Selectivity $K_i(\delta)/K_i(\mu)$
Tyr-D-Ala-Gly-N-MePhe-Gly-ol	None	DAMGO	4.13 ± 0.84	2540 ± 155	> 10 000	52.3
Tyr-c[D-Pen-Gly-Phe-D-Pen]OH	S–S	DPDPE	810 ± 66	3.98 ± 0.46	> 10 000	0.005
Tyr-c[D-Cys-Phe-D-Pen]NH_2	S–S	JOM-5	7.01 ± 0.45	58 ± 4.7	> 10 000	8.23
Tyr-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	JOM-6	0.29 ± 0.04	24.8 ± 1.46	2000 ± 235	83.3
L-*t*-Hpp-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**1**	0.319 ± 0.03	27.3 ± 1.0	615 ± 187	85.6
L-Hat-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**2**	0.401 ± 0.08	23.7 ± 1.3	933 ± 237	59.1
D-Hat-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**3**	0.392 ± 0.04	57.9 ± 3.8	281 ± 45.2	148
Tyr-c[D-Cys-Δ^E-Phe-D-Pen]NH_2	S–S	**4**	8.74 ± 1.15	161 ± 19.7	2270 ± 320	18.4

a. Bridge between the second and fourth amino acids (or second and fifth amino acids in the case of DPDPE). S–Et–S denotes -S–CH_2–CH_2–S-.

plane but are ≈ 2.5 Å apart, and as a consequence there is also a difference in the position of the hydroxyl groups (Fig. 2). A model for the binding of ligands to the mu opioid receptor (16) proposes that the quaternary nitrogen of the amine group interacts forming an ion pair with the carboxylic acid group of the Asp^{147} residue in the third transmembrane domain, while the oxygen atom of the tyramine hydroxyl group forms a hydrogen bond interaction with the hydrogen attached to the secondary nitrogen of the imidazole moiety of the His^{297} residue in the sixth transmembrane domain. Assuming that both peptides occupy the binding site in broadly the same orientation, this difference in position of the tyramine phenolic rings of **2** and **3** requires that the hydroxyl groups of both can form hydrogen bonds with His^{297} (Fig. 2) and that the hydrophobic residues comprising the binding pocket, namely well-conserved hydrophobic residues in the fifth (Ile^{234}), sixth (Ile^{296} and Val^{300}) and seventh (Cys^{321}) transmembrane domains, are flexible enough to be able to accommodate both L- and D-Hat in position 1 of these tetrapeptides.

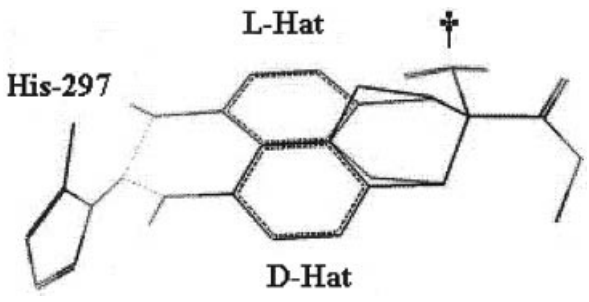

Figure 2. Overlap of L-Hat and D-Hat showing hypothetical hydrogen bonding to the His^{297} residue in the sixth transmembrane domain of the mu opioid receptor. † denotes the position of the primary nitrogen in both **3** and **4**.

Peptide **4** is the dehydro(E)phenylalanine analog of JOM-5, Tyr-c[D-Cys-Δ^EPhe-D-Pen]NH_2. The Δ^E-Phe^3 residue introduces a double bond between the α and β carbons, restricting rotation of the Cα–Cβ, but not Cβ–Cγ, bonds. This peptide exhibited affinity for the mu opioid receptor similar to that of JOM-5, but with an almost 3-fold decrease in delta receptor affinity. This suggests that the delta, but not mu, binding conformation is energetically unfavorable in **4**, implying that different conformations of the side chain of the Phe^3 residue are required for binding to mu (*gauche*) and delta (*trans*) opioid receptors. Indeed, we have previously shown in a tetrapeptide series based on JOM-13 that binding to the delta opioid receptor requires a *gauche* orientation of the Phe^3 side chain, as in the Δ^Z-Phe^3 analog (17) and in a group of four β-methylphenylalanine isomers (4). In contrast, binding to the mu receptor requires a *trans* orientation of the Phe^3 side chain, as seen in the Δ^E-Phe^3 analog (18).

Peptidergic opioids related to enkephalin, such as this series of tetrapeptides, traditionally exhibit very low affinity for the kappa opioid receptor. All of the peptides tested here showed low affinity for the kappa opioid receptor, as measured by displacement of [^{3}H]U69,593, with the exception of peptide **3**. This analog showed moderate-to-low affinity, ≈ 280 nM, but this is still higher than most peptides of this type. Consequently, the peptides tested all showed selectivity for mu and delta over kappa. For example, JOM-6 exhibited almost 7000-fold selectivity for the mu over the kappa opioid receptor and 80-fold selectivity for delta over kappa. It is apparent that those peptides containing the -S–CH_2–CH_2-S- ring closure exhibit higher affinity for the kappa opioid receptor than those with the -S–S- linkage, implying that conformational freedom of

the peptide ring system is important in allowing for correct orientation within the binding pocket of the kappa opioid receptor.

GTPγS binding assays

The potencies and relative efficacies of the cyclic peptides for the mu opioid receptors are shown in Table 3 and compared with values for DPDPE and DAMGO.

The [^{35}S]-GTPγS assay provides a functional measure of the efficacy of a ligand interacting with a G-protein-coupled receptor (5). In this assay, performed in membranes from C_6μ cells, all of the peptides tested produced stimulation equal to or greater than that produced by a maximal concentration of the mu opioid agonist fentanyl. Indeed, as shown in Table 3, peptides JOM-6 and **1** produced stimulation significantly greater than fentanyl, suggesting very high efficacy. Analogs **2** and **3** exhibited potencies ≈17- and 6-fold higher, respectively, than DAMGO. Peptide **1** showed potency comparable with that of DAMGO, while peptide **4** exhibited very low potency but still a full maximal response.

The conformational restrictions imposed on the aromatic moiety of residue 1 in peptides **1**, **2** and **3** had a greater effect on relative potency in the [^{35}S]-GTPγS assay than on affinity as measured by the ligand binding assay. For example, **2** exhibited a 7-fold higher potency than JOM-6 and **1** showed a 3-fold lower potency in the [^{35}S]-GTPγS assay, while both had equal affinity to JOM-6 in the ligand binding assay. Peptide **4** was 60-fold less potent at the mu opioid receptor than JOM-5, but still gave a full maximal response. However, **4** displayed almost equivalent affinity to JOM-5, suggesting that although all of the peptides tested produced maximal stimulation in this assay, their true efficacies at the mu opioid receptor are different. The L- and D-Hat isomers **2** and **3** produced a similar maximal effect and only 3-fold differing potency in the [^{35}S]-GTPγS assay, confirming that the complementary feature within the opioid binding site has the degree of conformational freedom necessary to bind both L- and D-isomers.

At the delta receptor, none of the peptides tested were able to produce stimulation of [^{35}S]-GTPγS equivalent to a maximal concentration of the nonpeptide delta receptor full agonist BW 373,U86. Maximal stimulation of binding ranged from 35.4% for **4** to 73.9% for **3**. Potencies were also more varied, and lower, than found in the same assay at the mu receptor, ranging from 57.5 nM (**1**) to 1550 nM (**3**), as was the case with their affinities determined by ligand binding assays. All of the peptides, except **4**, exhibited greater potency and efficacy in the [^{35}S]-GTPγS assay at the mu receptor than at the delta receptor, with functional selectivity ranging from 7-fold (**1**) to 1060-fold (**3**). As with the mu receptor, the relationship between affinity and potency is not parallel for all the compounds in the series. For example, peptide **1** exhibited higher potency than JOM-6 at the delta receptor, despite showing equivalent affinities. Also, **2** and **3** had similar affinities at the delta opioid receptor, but 6-fold different potencies in the [^{35}S]-GTPγS assay. This differentiation resulted in the D-Hat[1] containing peptide **3** becoming functionally very selective for the mu over delta opioid receptors, with a 1060-fold preference for mu as measured using the [^{35}S]-GTPγS assay.

In conclusion, we have identified a peptide, D-Hat-c[D-Cys-Phe-D-Pen]NH_2 (Et) (**3**), with high potency and >1000-fold functional selectivity for the mu over the

Table 3. **Potency and relative efficacy as measured in the[^{35}S]-GTPγS assay**

			mu		delta	
Peptide structure Amino acid sequence	Bridge[a]	Analog name	EC_{50} (nM)	max (%)[b]	EC_{50} (nM)	max (%)[b]
Tyr-D-Ala-Gly-N-MePhe-Gly-ol	none	DAMGO	6.4 ± 1.45	110.0 ± 4.6	>10 000	–
Tyr-c[D-Pen-Gly-Phe-D-Pen]OH	S–S	DPDPE	n.d.[c]	n.d.[c]	3.0	102.7
Tyr-c[D-Cys-Phe-D-Pen]NH_2	S–S	JOM-5	17.8 ± 3.56	98.2 ± 2.3	n.d.[c]	n.d.[c]
Tyr-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	JOM-6	2.93 ± 0.83	125.8 ± 6.0[d]	337.7 ± 72.6	72.6 ± 1.9[d]
L-*t*-Hpp-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**1**	8.76 ± 1.99	125.7 ± 5.1[d]	57.5 ± 3.9	40.9 ± 1.0[d]
L-Hat-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**2**	0.40 ± 0.21	102.1 ± 5.5	247.7 ± 37.8	69.6 ± 5.1[d]
D-Hat-c[D-Cys-Phe-D-Pen]NH_2	S–Et–S	**3**	1.44 ± 0.36	109.6 ± 5.4	1550 ± 117.2	73.9 ± 3.2[d]
Tyr-c[D-Cys-Δ^E-Phe-D-Pen]NH_2	S–S	**4**	1060 ± 69.1	117.4 ± 5.6[d]	567.6 ± 61.7	35.4 ± 8.8[d]

a. Bridge between the second and fourth amino acids (or second and fifth amino acids in the case of DPDPE). S–Et–S denotes -S–CH_2–CH_2–S-. b. Values represent percentage of the maximal response to 10 μM fentanyl (mu) or BW 373,U86 (delta). c. n.d., not determined. d. $P<0.05$ (Student's *t*-test) compared with the maximal response to 10 μM fentanyl (mu) or BW 373,U86 (delta).

delta opioid receptor. Moreover, we have demonstrated that the phenolic ring of the first residue of opioid peptides may have a degree of positional flexibility without detriment to binding at the opioid receptors, and that structural requirements for binding to the mu or delta opioid receptor are not necessarily the same as the structural requirements for activation of the receptor. Thus, variations in affinity are not matched by parallel changes in potency. This is perhaps illustrated most graphically by the *trans*-hydroxyphenylproline derivative **1** and the L-Hat analog **2**, which displayed comparable affinities at the mu receptor but 20-fold different potencies, and suggests that the structural requirements for efficacy and affinity are different, highlighting the need to measure relative efficacy in SAR studies.

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