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Further studies of tyrosine surrogates in opioid receptor peptide ligands

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Abstract—A series of opioid peptide ligands containing modified N-terminal tyrosine (Tyr) residues was prepared and evaluated against cloned human μ, δ, and κ opioid receptors. This work extends the recent discovery that (*S*)-4-carboxamidophenylalanine (Cpa) is an effective tyrosine bioisostere. Amino acids containing negatively charged functional groups in place of tyrosine's phenolic hydroxyl lacked receptor affinity, while exchange of Tyr for (*S*)-4-aminophenylalanine was modestly successful. Peptides containing the new amino acids, (*S*)-4-carboxamido-2,6-dimethylphenylalanine (Cdp) and (*S*)-β-(2-aminobenzo[*d*]thiazol-6-yl)alanine (Aba), displayed binding (K_i) and functional (EC_{50}) profiles comparable to the parent ligands at the three receptors. Cdp represents the best performing Tyr surrogate in terms of overall activity, while Cpa and Aba show a subtle proclivity toward the δ receptor.

Opioid peptide ligands continue to be of interest as pharmacological tools for advancing opioid receptor biology and as potential therapeutic agents.[1] The discovery of endogenous opioid peptides,[2] principally the enkephalins, initiated an intense search for potent, selective ligands for the μ, δ, and κ opioid receptor types.[3] Examples of such ligands, derived from either natural sources or de novo synthesis, include DPDPE[4a] and DSLET[4b] (δ selective), endomorphins[4c], and DAMGO[4d] (μ selective) and the dynorphins[4e] (κ selective). The N-terminal tyrosine residue [Tyr^1] is universally found in the expansive family of opioid peptide ligands.[3a] It satisfies a minimal pharmacophore model characterized by an appropriately spatially oriented basic nitrogen and a hydroxylated phenyl ring.[3a,b] These two binding elements constitute the putative message of the message-address concept of opioid ligand–receptor interaction.[5] SAR studies conducted more than 25 years ago demonstrated that the Tyr amino group may be alkylated yielding peptide analogs that retain potency. Extensive structure–activity relationship (SAR) studies conducted over the past two decades have demonstrated that the Tyr amino group may be alkylated, yielding peptide analogs that retain potency.[6a,b] *N*-Acylation or exchange of the amino group for a hydrogen atom or a methyl group can result in the conversion of agonists to antagonists.[6c–h] In contrast, removal or derivatization of the tyrosine phenolic hydroxyl group leads to a loss in opioid receptor binding affinity.[3] Replacement of tyrosine with phenylalanine or tyrosine *O*-methyl ether gives rise to peptides with 100- to >1000-fold decreased activity relative to the parent ligands. These and other SAR studies suggest an apparent immutable quality of the phenol hydroxyl.

We recently disclosed a highly effective amino acid surrogate for the terminal tyrosine residue.[7] The surrogate, (*S*)-4-carboxamidophenylalanine (Cpa) **3**, possesses a primary carboxamide (–$CONH_2$) group in place of the tyrosine phenolic OH. A selection of classical opioid peptide analogs were prepared with a [Tyr^1] → [Cpa^1] modification.[7] As exemplified by the [Leu^5]-enkephalinamide pair **1** and **2**, the Cpa-containing peptides consistently displayed binding affinities (K_i) comparable to the tyrosine reference peptides at the three cloned human μ, δ, and κ opioid receptors. The [Cpa^1] peptides retained agonist potency (EC_{50}) as measured by their

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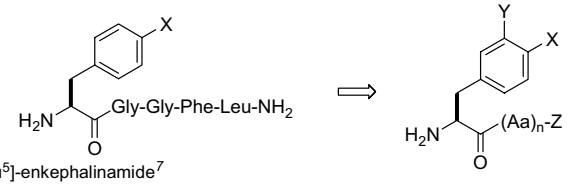
ability to stimulate the binding of [^{32}S]GTPγS to the cloned human receptors. The [Cpa^1] congeners of δ selective peptides generally showed enhanced δ receptor selectivity relative to the μ receptor. Reports by Weltrowska[8] and Breslin[9] have likewise documented the utility of 4-carboxamidophenylalanine derivatives as tyrosine bioisosteres in opioid peptides. In this report, the effectiveness of **3** is further demonstrated by incorporating the amino acid into three additional acyclic and cyclic peptides. Six other amino acids **4**–**9** are surveyed as potential surrogates for the N-terminal Tyr residue in a series of representative opioid peptides **i**.[10]

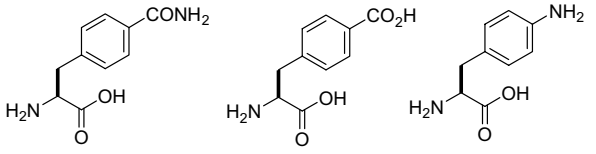
Tyrosine phenolic OH to $CONH_2$ exchange in opioid peptide ligands.



1: [Leu^5]-enkephalinamide[7]
X = OH; K_i = 25 nM (μ); 5.3 nM (δ); 440 nM (κ)
2: [Cpa^1-Leu^5]-enkephalinamide
X = $CONH_2$; K_i = 43 nM (μ); 3.3 nM (δ); 240 nM (κ)

i: opioid peptide ligands with modified [Tyr^1]

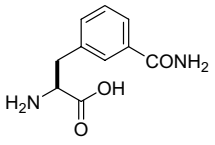
[Tyr^1] replacements investigated in this study:



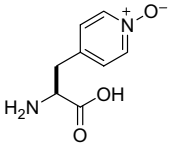
3: (*S*)-4-Carboxamido-phenylalanine (Cpa)

4: (*S*)-4-Carboxy-phenylalanine (Cxp)

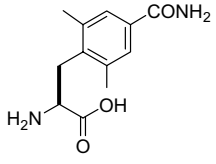
5: (*S*)-4-Aminophenyl-alanine (Apa)



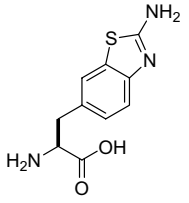
6: (*S*)-3-Carboxamido-phenylalanine (mCpa)



7: (*S*)-β-(4-Pyridinyl-1-oxide)-alanine (Poa)



8: (*S*)-4-Carboxamido-2,6-dimethylphenylalanine (Cdp)



9: (*S*)-β-(2-Aminobenzo[*d*]thiazol-6-yl)alanine (Aba)

As an extension of our previous work,[7] [Met^5]-enkephalin **10** was targeted for modification with amino acid **3**. Enkephalin **10** is an opioid peptide modestly selective for the δ versus μ receptor. In the cloned human receptor binding assays **10** displayed K_i = 19 nM (μ), 2.0 nM (δ), and >1000 nM (κ); μ/δ = 9.5. Replacement of [Tyr^1] in **10** with **3** furnished peptide **11**, [Cpa^1, Met^5]-enkephalin. Binding affinity and selectivity obtained for **11** reveals that it was nearly equivalent to the parent ligand: K_i = 28 nM (μ), 1.8 nM (δ), and >1000 nM (κ); μ/δ = 16. In the [^{35}S]GTPγS functional assay, **11** was 9-fold more potent as a δ agonist: EC_{50} = 5.2 nM for **11** versus 47 nM for **10**. The corresponding amide **13**, [Cpa^1, Met^5]-enkephalinamide, was then synthesized and compared to [Met^5]-enkephalinamide **12**. The K_i and EC_{50} values for the δ receptor were significantly enhanced in the [Cpa^1] analog **13** (K_i = 10 and 1.3 nM; EC_{50} = 300 and 10 nM; **12** and **13**, respectively) with a modest 12-fold improvement in selectivity over the μ receptor (μ/δ = 1.2 for **12** vs 14 for **13**). The affinity for **13** (K_i = 460 nM) at the κ receptor was approximately 2-fold that of **12**. The Cpa derivative **15** of the well-known selective δ ligand DPDPE **14** was also prepared. The activity and δ selectivity of this peptide pair was again comparable (K_i = 3.2 nM (**14**) vs 19 nM (**15**)). This result is in agreement with the recent work of Schiller and coworkers,[8] who reported that the Cpa derivative of the cyclic enkephalin analog, H-Tyr-c[cys-Gly-Phe(*p*-NO_2)-cys]NH_2, (IC_{50} = 19 pM, mouse vas deferens (δ receptor enriched)) displayed subnanomolar potency in the mouse vas deferens assay (IC_{50} = 232 pM). The binding and functional data obtained for peptide pairs **10/11**, **12/13**, and **14/15** are consistent with previous results employing **3** as a surrogate for [Tyr^1].[7]

Homology modeling and site-directed mutagenesis studies suggest that the tyrosine OH in the peptide (and non-peptide) ligands may form a hydrogen bond to a lysine ε-amino group or a histidine NH group in the putative binding cavity of the δ receptor.[11] It is therefore conceivable that a negatively charged carboxylate could potentially engage the basic active-site residues through either hydrogen bonding or a productive charge–charge interaction. For this reason, [Tyr^1] in the selective δ peptide DADLE **16** (K_i = 0.89 nM; μ/δ = 39) was exchanged with (*S*)-4-carboxyphenylalanine (Cxp) **4**. The new peptide analog **19** was found to be weakly active at δ with a micromolar K_i value.[12] DADLE analog **20**, containing the (*S*)-pyridinyl-1-oxide amino acid (Poa) **7**,[10b], was explored as a potential tyrosine mimic ([Tyr^1] → [Poa^1]). This analog, possessing a more compact negative point charge, was devoid of activity. [Poa^1]-containing peptides **26** and **32** were weakly active. The suitability of (*S*)-4-aminophenylalanine **5** was then examined in the DADLE series (**16** vs **18**). In this case, the anilino moiety was reasonably tolerated by the δ receptor (K_i = 19 nM (**18**); 0.89 nM (**16**) in contrast to the Cxp and Poa analogs. The aniline group was less tolerated at the μ receptor, where **18** exhibited a 40-fold decrease (K_i = 1400 nM vs 35 nM) in affinity for this receptor.

The next mini series of peptides scrutinized were those containing a (*S*)-3-carboxamidophenylalanine (mCpa) **6**. The [Tyr^1] in [Leu^5]-enkephalin, DADLE, and DSLET was replaced with **6** to yield the corresponding peptides **21**, **27**, and **33**, respectively. This was carried out for three reasons. First, a N-terminal *m*-tyrosine residue has been shown to possess modest affinity for the opioid receptors.[13] Second, Sperlinga and coworkers found 6-hydroxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylate to be an effective constrained tyrosine mimetic.[14] The corresponding 7-hydroxy isomer lost its affinity for the μ receptor but δ receptor affinity was preserved. Third, the primary carboxamide as a direct

replacement of phenol in nonpeptide opioid ligands has been thoroughly documented.[15] However, the $CONH_2$ at other positions in the central aromatic ring of these ligands has not been explored, perhaps due to a lack of synthetic accessibility. Hence, the *meta*-disposed $CONH_2$ in **6** could potentially present an opportunity to discover alternative hydrogen-bonding interactions between the opioid receptors and these modified ligands. Unfortunately, this was not the case. Peptides **21**, **27**, and **33** all experienced a minimum of a 10-fold loss in binding at both μ and δ relative to the parent ligands **16**, **24**, and **30**.[16]

Returning to (*S*)-4-carboxamidophenylalanine **3** as a favored surrogate, the SAR in the series was explored further by introducing methyl groups at the 2- and 6-positions of the aromatic ring. The resulting amino acid Cdp **8** is a direct analog of 2,6-dimethyltyrosine (Dmt). Dmt imparts greater potency and μ selectivity relative to tyrosine in a range of peptide ligands.[17] This is presumably due to a combination of the greater lipophilicity of the amino acid and a favorable conformational bias of the aromatic ring imposed by the methyl groups flanking the β-carbon of the side chain. Peptides **16**, **24**, and **30** were again targeted for modification with **8**, affording the new peptides [Cdp^1]-DADLE **22**, [Cpd^1, Leu^5]-enkephalin **28**, and [Cdp^1]-DSLET **34**. [Cdp^1] proved to be a surrogate for tyrosine. The binding constants for **22**, **28**, and **34** against δ were equivalent to the parent ligands: K_i (δ) **28/24** = 1.0 nM/1.1 nM; **22/16** = 0.78 nM/0.89 nM; **34/30** = 1.2 nM/1.1 nM. Equipotent EC_{50} values were also observed for the peptide pairs. As noted previously, Cpa had a tendency to skew μ/δ selectivity in favor of the δ opioid receptor by a factor of 2- to 50-fold in several [Tyr^1]/[Cpa^1] peptide pairs. Cdp showed no such tendency. In all three [Cdp^1]-peptides, the μ binding affinity exceeded those values determined for the parent peptides: K_i (μ) **28/24** = 9.8 nM/50 nM; **22/16** = 4.3 nM/35 nM; **34/30** = 19 nM/140 nM. The corresponding μ/δ ratios were 9.8 versus 45 for **28/24**, 5.5 versus 39 for **22/16**, and 16 versus 130 for **34/30**. The data are in agreement with the opioid recep-

Table 1. In vitro binding and functional data for opioid peptides with [Tyr^1] replacements

Peptide sequence (No.)	Ligand	K_i[a] (nM)				EC_{50}[b] (nM)	
		μ	δ	κ	μ/δ	μ	δ
H-Tyr-Gly-Gly-Phe-Met-OH (**10**)	Met-enkephalin	19	2.0	[c]	9.5	620	47
H-Cpa-Gly-Gly-Phe-Met-OH (**11**)		28	1.8	1030	1.6	140	5.2
H-Tyr-Gly-Gly-Phe-Met-NH_2 (**12**)	Met-enkephalinamide	12	10	210	1.2	630	300
H-Cpa-Gly-Gly-Phe-Met-NH_2 (**13**)		18	1.3	460	14	270	10
H-Tyr-c[pen-Gly-Phe-pen]OH (**14**)	DPDPE	[c]	3.2	[c]	—	[d]	21
H-Cpa-c[pen-Gly-Phe-pen]OH (**15**)		[c]	19	[c]	—	[d]	320
H-Tyr-ala-Gly-Phe-leu-OH (**16**)[e,f]	DADLE	35	0.89	[c]	39	330	12
H-Cpa-ala-Gly-Phe-leu-OH (**17**)[e]		120	8.2	[c]	15	640	130
H-Apa-ala-Gly-Phe-leu-OH (**18**)		1400	19	[c]	75	[d]	74
H-Cxp-ala-Gly-Phe-leu-OH (**19**)		[c]	1100	[c]	—	[d]	[d]
H-Poa-ala-Gly-Phe-leu-OH (**20**)		[c]	[c]	[c]	—	[d]	[d]
H-mCpa-ala-Gly-Phe-leu-OH (**21**)		560	170	[c]	3.3	[d]	1700
H-Cdp-ala-Gly-Phe-leu-OH (**22**)		9.8	0.78	2400	5.5	49	2.5
H-Aba-ala-Gly-Phe-leu-OH (**23**)		1200	10	[c]	29	[d]	150
H-Tyr-Gly-Gly-Phe-Leu-OH (**24**)[e]	Leu-enkephalin	50	1.1	[c]	45	140	13
H-Cpa-Gly-Gly-Phe-Leu-OH (**25**)[e]		110	1.9	[c]	58	[c]	15
H-Poa-Gly-Gly-Phe-Leu-OH (**26**)		[c]	[c]	[c]	—	[d]	[e]
H-mCpa-Gly-Gly-Phe-Leu-OH (**27**)		[c]	475	[c]	—	[d]	[e]
H-Cdp-Gly-Gly-Phe-Leu-OH (**28**)		9.8	1.0	1300	9.8	180	4.1
H-Aba-Gly-Gly-Phe-Leu-OH (**29**)		1200	19	[c]	63	[d]	580
H-Tyr-ser-Gly-Phe-Leu-Thr-OH (**30**)[e]	DSLET	140	1.1	[c]	130	150	5.1
H-Cpa-ser-Gly-Phe-Leu-Thr-OH (**31**)[e]		370	1.2	[c]	308	[d]	9.9
H-Poa-ser-Gly-Phe-Leu-Thr-OH (**32**)		[c]	360	[c]	—	[d]	[d]
H-mCpa-ser-Gly-Phe-Leu-Thr-OH (**33**)		1100	42	[c]	26	[d]	730
H-Cdp-ser-Gly-Phe-Leu-Thr-OH (**34**)		19	1.2	[c]	16	250	3.5
H-Aba-ser-Gly-Phe-Leu-Thr-OH (**35**)		640	3.8	[c]	170	[d]	97
H-Tyr-ala-Gly-Phe-Met-OH (**36**)[e]	[ala^2]-Met-enkephalin	14	0.35	[c]	40	210	1.0
H-Cpa-ala-Gly-Phe-Met-OH (**37**)[e]		110	0.72	[c]	150	320	1.4
H-Aba-ala-Gly-Phe-Met-OH (**38**)		100	1.0	[c]	100	350	24

[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.[20] K_i values are the geometric means computed from at least three separate determinations.

[b] The potencies (EC_{50}) of the peptides were determined by testing the ability of a range of concentrations of each peptide to stimulate the binding of [^{35}S]GTPγS to cloned human μ, δ, κ opioid receptors expressed in separate cell lines.[20] EC_{50} values are the geometric means computed from at least three separate determinations.

[c] K_i or EC_{50} estimated to be >10,000 nM.

[d] Not determined.

[e] See Ref. 7.

[f] D-Amino acids are indicated by all lower case letters.

tor binding data (rat) reported by Breslin for a related series of Cdp-containing phenylimidazoles.[9]

2-Aminothiazole has been employed as a heterocyclic bioisostere of the phenol moiety in dopamine agonists[18a] and the antiparkinsonian agent pramipexole.[18b] Neumeyer and coworkers recently applied the modification to the morphinan and benzomorphan classes of opiates.[19] It is apparent that the 2-amino group of the heterocycle does not overlay directly onto the phenolic OH of Tyr or the carboxamide NH_2 of Cpa, but rather extends further into the hydrogen-bonding region of the receptor. It was therefore of interest to incorporate (*S*)-2-aminobenzothiazoyl alanine (Aba) **9** into opioid peptides and compare their behaviors relative to the parent ligands and [Cpa^1] analogs. Several [Aba^1] peptides were prepared and evaluated against the cloned human receptors. These included peptides derived from DADLE (**16** → **23**), [Leu^5]-enkephalin (**24** → **29**), DSLET (**30** → **35**), and [ala^2, Met^5]-enkephalin (**36** → **38**). As shown in Table 1, all of the [Aba^1] peptides bind to both the δ and μ receptors, demonstrating a proclivity toward δ. Their binding and selectively profiles were more similar to Cpa than Tyr: [Aba^1]-DADLE K_i = 10 nM, δ and 290 nM, μ; [Cpa^1]-DADLE K_i = 8.2 nM, δ and 120 nM, μ; DADLE K_i = 0.89 nM, δ and 35 nM, μ. The most potent and selective Aba analog was [Aba^1, ala^2, Met^5]enkephalin **38**. It possessed a K_i = 1.0 nM, a μ/δ ratio = 100 and potent agonist action (EC_{50} = 24 nM).

In summary, Cpa **3** was successfully incorporated into [Met^5]-enkephalin and DPDPE, further demonstrating the capacity of this unique amino acid as a surrogate for [Tyr^1] in opioid peptides. In addition, a survey of six other potential amino acid surrogates was carried out. Amino acid derivatives carrying a negative charge, for example **4** and **7**, lacked affinity for the cloned human opioid receptors, despite their potential to engage in a favorable charge–charge interaction with putative basic hydrogen-bonding residues in the active site. Amino acid **6**, a regioisomer of **3**, resulted in peptides with muted opioid receptor affinity. The 2,6-dimethyl analog of **3**, Cdp **8**, displayed equivalent binding against δ and an enhanced affinity for the μ receptor relative to the Tyr reference peptides. Aba **9** possessed a binding and functional profile comparable to Cpa. Amino acids Cpa, Cdp, and Aba are all reasonably effective surrogates for [Tyr^1]. Cdp represents the best performing surrogate in terms of overall binding affinity, while Cpa and Aba are more readily accommodated by the δ receptor.

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10. (a) Boc-protected amino acids **3**–**6**, **8**–**9** are commercially available from RSP Amino Acids, Boston, MA; (*S*)-β-(4-pyridinyl-1-oxide)alanine **7** was prepared by the method of Cushman: (b) Cushman, M.; Chinnasamy, P.; Chakraborti, A. k.; Jurayj, J.; Geahlen, R. L.; Haugwitz, R. D. *Int. J. Pept. Protein Res.* **1990**, *36*, 538; (c) The modified peptides in Table 1 were prepared on Wang or Rink resin using classical Fmoc chemistry with commercially available appropriately protected amino acids wherein the *N*-Boc protected **3**–**9** were used in the final coupling step. Acid-mediated cleavage of the penultimate resin resulted in simultaneous peptide release and Boc-deprotection. Peptides were purified to >98% purity by HPLC and exhibited physical and spectroscopic properties consistent with their structure.
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12. Accordingly, Cxp and Apa were substituted for [Tyr^1] in cyclic opioid peptide analogues (see Ref. 8). In this case,

[Cxp[1]] was 39,000-fold and [Apa[1]] was 640-fold less active than its [Tyr[1]] parent.
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16. A 37-fold decrease in κ receptor binding was observed for a κ selective peptide modified with **6**: K_i for [mCpa[1]]-dynorphin (1–11) = 9.8 nM (κ), 3563 nM (μ), 1514 nM (δ) versus K_i for dynorphin (1–11) = 0.26 nM (κ), 61 nM (μ), 9.1 nM (δ). For the [Cpa[1]]-dynorphin (1–11) binding data, see Ref. 7.
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