Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 1269–1272

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Dermorphin Tetrapeptide Analogues with 2′,6′-dimethylphenylalanine (Dmp) Substituted for Aromatic Amino Acids have High μ Opioid Receptor Binding and Biological Activities[1]

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Received 9 September 2002; accepted 22 January 2003

Abstract—To investigate the value of the 2′,6′-dimethylphenylalanine (Dmp) residue as an aromatic amino acid substitution, we prepared analogues of the μ opioid receptor-selective dermorphin tetrapeptide Tyr-D-Arg-Phe-βAla-NH_2 (YRFB) in which Dmp or its D-isomer replaced Tyr^1 or Phe^3. Replacing Phe^3 with Dmp essentially tripled μ receptor affinity and the receptor's in vitro biological activities as determined with the guinea pig ileum (GPI) assay but did not change δ receptor affinity. Despite an inversion of the D configuration at this position, μ receptor affinity and selectivity remained comparable with those of the L-isomer. Replacing the N-terminal Tyr residue with Dmp produced a slightly improved μ receptor affinity and a potent GPI activity, even though the substituted compound lacks the side chain phenolic hydroxyl group at the N-terminal residue. Dual substitution of Dmp for Tyr^1 and Phe^3 produced significantly improved μ receptor affinity and selectivity compared with the singly substituted analogues. Subcutaneous injection of the two analogues, [Dmp^3]YRFB and [Dmp^1]YRFB, in mice produced potent analgesic activities that were greater than morphine in the formalin test. These lines of evidence suggest that the Dmp residue would be an effective aromatic amino acid surrogate for both Tyr and Phe in the design and development of novel opioid mimetics.

A Tyr residue at position 1 and a Phe residue at position 3 or 4 of opioid peptides are important structural elements for their interaction with opioid receptors. In studies of structure–activity relationships of the opioid peptide, 2′,6′-dimethyltyrosine (Dmt) has played a key role in the design of opioid mimetics with high receptor affinities and biological activities including the Dmt-Tic pharmacophore peptides, which are potent δ opioid receptor antagonists.[2–7] The substitution of Dmt at position Tyr^1 of opioid peptides generally produces considerably improved receptor affinity and biological activity,[8–14] although the relationship between the opioid receptors and the Dmt residue of the peptides is not well understood. Modifying opioid peptides by dimethylating the Phe aromatic ring may be a fruitful approach to the design of receptor-specific opioid mimetics. We therefore substituted the 2′,6′-dimethylphenylalanine (Dmp) residue for Phe. A modified Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) with Dmp substituted for Phe^4 exhibited activities towards μ and δ receptors that were lower than those of the parent peptide.[15] However, dual substitutions of D-Dmp^4 and Dmt^1 produced an analogue with potent μ receptor antagonist activity but weak δ receptor antagonist activity.[15] In contrast with the results obtained with the enkephalin analogues, substitution of the Dmp residue for Phe at position 3 of two heptapeptides, μ receptor-selective dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and δ receptor-selective deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2), resulted in significant improvements in both preferential receptor affinity and selectivity.[16] Consequently, we have further investigated in other opioid peptides the effects of substituting the Dmp residue for aromatic amino acids.

The present study investigates the substitution of the L- or D-Dmp residue for the Tyr^1 and Phe^3 residues of the μ receptor-selective dermorphin tetrapeptide Tyr-D-Arg-Phe-βAla-NH_2 (YRFB)[17] and describes novel aspects of the new analogues' interactions with opioid receptors (Fig. 1).

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0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00110-0

Table 1. Opioid receptor binding affinities and in vitro biological activities of YRFB analogues

Peptides	Receptor binding affinity K_i (nM)[a]		δ/μ ratio	In vitro bioassay IC_{50} (nM)[a]		MVD/GPI ratio
	[^{3}H]DAMGO (μ)	[^{3}H]deltorphin II (δ)		GPI (μ)	MVD (δ)	
[Dmp^3]YRFB (**1**)	0.0350±0.0167	544±143	15,543	1.67±0.24	27.9±5.0	16.7
[D-Dmp^3]YRFB (**2**)	0.0618±0.0109	>2823	>45,679	19.8±1.9	305±53	15.4
[Dmp^1]YRFB (**3**)	0.0623±0.0140	2572±947	41,284	9.88±1.04	188±52	19.0
[D-Dmp^1]YRFB (**4**)	7.62±1.75	>2823	>370	320±30	1474±283	4.6
[Dmp^{1,3}]YRFB (**5**)	0.0216±0.0062	1688±458	78,148	2.76±0.56	50.1±8.6	18.2
[Phe^1]YRFB (**6**)	7.17±1.03	>2823	>393	633±89	7143±950	11.2
YRFB	0.172±0.025	482±121	2802	5.31±0.72	116±18	21.8
[Dmt^1]YRFB[b]	0.00205±0.00069	1.13±0.13	551	0.034±0.065	0.398±0.085	11.7

[a]Values are means of 4–8 experiments±standard deviation.
[b]Data cited are from ref 18.

Results and Discussion

All analogues were synthesized by solid-phase Fmoc chemistry as described.[18] Peptides were constructed on a solid support by using Fmoc-Dmp racemate; diastereoisomers were then separated by preparative HPLC.[16,19]

We determined the receptor-binding affinity of peptides by using rat brain synaptosomal fractions. The in vitro biological activities of analogues with respect to μ and δ receptors were evaluated as the ability to inhibit electrically induced contractions of guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations, respectively. GPI tissue contains predominantly μ receptors, whereas MVD tissues mainly include δ receptors.[20] Results of the receptor binding and in vitro biological activity experiments with the modified peptides are presented in Table 1. Substituting Dmp for Phe^3 in YRFB produced analogue **1**, which exhibited about a 5-fold increase in μ receptor affinity but no significant change in δ receptor affinity, compared with the parent peptide. The biological activity of this compound in the GPI assay coincided well with the binding data, but the activity in the MVD assay was slightly increased. These results indicated that Dmp is as effective a Phe surrogate for improving μ receptor affinity and selectivity of YRFB as it is for the dermorphin and deltorphin heptapeptides.[16] In contrast to the results with dermorphin, inverting the D configuration at this position produced analogue **2**, which exhibited a μ receptor affinity as high as parent compound YRFB but with a slightly lower GPI potency. Replacing Tyr at position 1 with Dmp (**3**) produced a significantly higher μ affinity and a considerably lower δ affinity than YRFB, and improved μ receptor selectivity by 15-fold. This compound, however, exhibited slightly lower GPI and MVD potensies than YRFB. The D-Dmp substitution for Tyr^1 (**4**) markedly reduced μ and δ receptor affinities and in vitro biological potencies, suggesting that the L-configuration at this position is crucial to receptor interactions. The dual substitutions of Dmp for aromatic amino acids at positions 1 and 3 produced analogue **5** with a binding affinity and selectivity for the μ receptor that were slightly improved relative to those of analogue **1** or **3**. This analogue also exhibited a slightly higher GPI potency than did YRFB, a result similar to that seen with analogue **1**. As shown in Table 2, the low K_e value (21.3–25.1 nM) with the μ receptor selective antagonist CTAP[21] in the GPI assay demonstrated CTAP inhibition of the high activity of Dmp-containing analogues (**1**, **3** and **5**) and suggest that the activity is mediated via μ opioid receptors. The low K_e values for CTAP in the MVD assay indicate its inhibition of these analogues' MVD activity but the δ receptor selective antagonist *N,N*(Me)$_2$Dmt-Tic-OH[22] did not inhibit this activity. This result may be due to μ receptors co-occurring in MVD tissue. Analogues **3** and **5** notably retained high μ

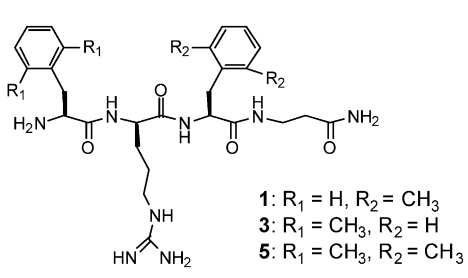

Figure 1. Dmp-containing dermorphin tetrapeptide analogues.

Table 2. K_e values of opioid receptor antagonists against Dmp-containing analogues as determined in the GPI and MVD assays

Peptides	K_e (nM)		
	GPI	MVD	
	CTAP	CTAP	N,N(Me)$_2$Dmt-Tic-OH
[Dmp^3]YRFB (**1**)	22.7	11.4	>1000
[Dmp^1]YRFB (**3**)	25.1	13.4	>1000
[Dmp^{1,3}]YRFB (**5**)	21.3	10.2	>1000
YRFB	26.8	21.2	>1000
[Dmt^1]YRFB	85.5	7.89	192
Deltorphin II	NT[a]	>1000	0.64

[a]Not tested.

Table 3. Antinociceptive activities of Dmp-containing YRFB analogues after subcutaneous injection in the formalin test

Peptides	ED_{50} (95% C. L.)[a] (nmol/kg)	
	First phase	Second phase
[Dmp^3]YRFB (**1**)	98.6 (26.7–364)	113 (48.6–264)
[Dmp^1]YRFB (**3**)	1946 (1026–3691)	1529 (1199–1950)
YRFB	628 (364–1280)	514 (378–700)
Morphine	3811 (2921–4973)	7319 (4058–13198)

[a]ED_{50} values and 95% confidence limits were determined according to Litchfield and Wilcoxon.[29]

receptor affinities and potent GPI activities despite the lack of a phenolic hydroxyl group in the side chain of the N-terminal residue, which is considered crucial for binding to and activating opioid receptors. However, some cyclic somatostatin- or DPDPE-based analogues have recently been reported to possess a high affinity for and/or potency with the μ receptor despite the absence of this group at the N-terminal residue. Such compounds include Phe-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]-NH_2,[23] D-Phe-c[Cys-Tyr-D-Trp-Orn-Thr-Pen]Thr-NH_2 (CTOP),[24] Phe-c[D-Cys-Phe-D-Pen]-NH_2(Et) (JH-54),[25,26] and Phe-c[D-Lys-Tyr-Trp].[27] Analogues **3** and **5** are examples of linear peptides lacking the N-terminal phenolic hydroxyl group but possessing high opioid activity. Our present results support the notion derived from studies of the interactions of the cyclic compounds that the Tyr hydroxyl moiety at the N-terminal residue of opioid peptides is not an absolute requirement for interaction with opioid receptors and signal transduction. Because replacing the Tyr^1 residue with Phe (analogue **6**) drastically reduced μ receptor affinity and GPI potency, the effects of the Dmp substitution on receptor interaction are mainly ascribed to enhanced hydrophobicity and/or an increased conformational stability of the dimethylated side chain of the aromatic ring due to reduced conformational freedom. The basic functional group of the D-Arg residue at position 2 may also be responsible for the potent receptor interaction, because the affinities of analogues **4** and **6** were very low; however, a significant affinity for the μ receptor was retained. In addition, the Dmt^1-substituted YRFB exhibited very high affinities for both the μ and δ receptors, which resulted in low receptor selectivity. Such trends have also been observed with other Dmt^1-substituted opioid peptides.[8,11,18] In contrast, substitution of Dmp^1 for Tyr^1 improved μ receptor selectivity exclusively, a result distinct from the effect of the Dmt^1 substitution.

Next, we tested analgesic activity of **1** and **3** with the formalin test[28] in mice and compared the results with those obtained with YRFB and morphine (Table 3). Subcutaneous injection of **1** or **3** produced dose-dependent analgesic activities in both the first phase and second phase. Analogue **1** exhibited activities about 40-fold and 70-fold more potent than morphine at the first phase and second phase, respectively. Analogue **3** also exhibited about 3-fold (first phase) and 5-fold (second phase) higher potencies than morphine, but the potencies were about 3-fold lower than those of YRFB. The analgesic potencies of these analogues correlated well with their GPI potencies (Table 1).

In conclusion, the present study demonstrated that the Dmp residue is an effective surrogate for both Tyr^1 and Phe^3 resides of the linear opioid peptide YRFB. The Dmp^1-substituted analogues of YRFB (**3** and **5**) produced significantly higher μ receptor affinities and selectivities than did the unmodified YRFB, despite the lack of an N-terminal phenolic hydroxyl group. The results with the Dmp residue therefore provide insights into the design and development of novel opioid mimetics.

Acknowledgements

We are grateful to Prof. S. Salvadori (University of Ferrara, Italy) for providing $N,N(Me)_2$Dmt-Tic-OH.

References and Notes

1. Amino acid and peptides are in the L configuration unless otherwise noted. Amino acid and peptide abbreviations used are those recommended by the IUPAC-IUB commission on Biochemical Nomenclature in: *Eur. J. Biochem.* **1984**, *139*, 9. Other abbreviations used are as follows: YRFB, Tyr-D-Arg-Phe-βAla-NH_2; Dmt, 2′,6′-dimethyltyrosine; Dmp, 2′,6′-dimethylphenylalanine; DAMGO, [D-Ala^2, $MePhe^4$, Gly-ol^5]-enkephalin; GPI, guinea pig ileum; MVD, mouse vas deferens, CTAP, D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2; Fmoc, N-9-fluorenylmethoxycarbonyl.
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