Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 57–62

BIOORGANIC & MEDICINAL CHEMISTRY

Rational design of dynorphin A analogues with δ-receptor selectivity and antagonism for δ- and κ-receptors

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Received 5 February 1997; accepted 14 August 1997

Abstract

Substitution of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) in place of Gly^2 in dynorphin A-(1-13)-NH_2 and -(1-11)-NH_2 (DYN) analogues (**1** and **2**) decreased the affinity to the κ, δ, and μ receptors, and κ selectivity. The analogue [D-Ala^2, des-Gly^3]DYN (**4**), a chimera between deltorphin/dermorphin N-terminal tripeptide and DYN, was virtually inactive for κ-sites while the affinities for δ- and μ-receptors remained essentially unchanged. The doubly substituted analogue [2′,6′-dimethyl-L-tyrosine (Dmt^1)-Tic^2]DYN (**3**) exhibited high δ-affinity ($K_i = 0.39$ nM) while μ- and κ-affinities were only an order of magnitude less (4–5 nM). Bioactivity of [Tic^2]DYN peptides (**1**–**3**) on guinea-pig ileum and rabbit jejunum revealed potent δ- and κ-antagonism, while the δ agonist potency of **4** was comparable to DYN. Thus, conversion from a κ-agonist to antagonist occurred with the inclusion of Tic into DYN analogues, similar to the appearance of antagonist properties with δ- and μ-opioid agonists containing a Tic^2 residue.

Key words: Dynorphin, peptide synthesis, bioassays, opioid peptides, opioid receptors.

1. Introduction

More than two decades ago enkephalins, endorphins, and dynorphins were recognized as the endogenous agonists for δ-, μ-, and κ-opioid receptors, respectively [1–4]; in addition, recently discovered endomorphins are highly selective μ-receptor ligands [5]. These receptors are inordinately sensitive to morphine and a variety of alkaloid-derived agonists and antagonists [6], which are traditionally applied extensively for the treatment of chronic [7,8] and acute [9] pain, as well as nonaddictive analogues in the current therapy regimes to combat alcohol dependency [10], are in use during immunosuppression in organ transplants [11] and in mediating the effects of narcotic addiction [12]. The relative importance of κ-opioid receptors in clinical applications was recently highlighted by the observation that female patients are more susceptible to analgesia by κ-opiate (nonpeptide) substances [13]. A major drawback in the application of peptide ligands in clinical studies involves both structural liability to enzymatic degradation and the inability to penetrate the blood–brain barrier. To overcome these deficits [1], considerable ingenious efforts have been made to analyse systematically the structure–activity relationships of selected opioid peptides including detailed examinations of the N-terminal message sequence (Tyr-Gly-Gly-Phe) in DYN [14] agonists. These linear [15,16] or conformationally constrained DYN analogues [17] exhibited enhanced selectivity for the κ_2-receptor. Discovery that the α-carbon in the second position in the amphibian-derived dermorphin/deltorphin peptides is in the D-stereoconfiguration [18] accounts for the enhanced biological stability [19]. Those observations led to extensive structure–activity

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0968-0896/98/$19.00
PII: S0968-0896(97)10008-6

studies on the side-chain requirements in the dermorphins and deltorphins primarily in their message (Tyr-D-Xaa-Phe) as well as address domains [20–23]. The β-casomorphin and morphiceptin opioid peptides contain a Pro^2 residue [24], identical to the endomorphins [5], however, they weakly interacted with opioid receptors. Nonetheless, these peptides might also have served as scaffolds for the synthesis of opioid analogues containing other cyclic resides, including Tic [22,23]. The Tic residue, which influences peptide bioactivity [22,23], is composed of two fused six member rings and a backbone dihedral torsion (ϕ) comparable to that observed with proline. The incorporation of Tic^2 in opioid agonists had profound consequences on receptor selectivity and bioactivity by the induction of δ antagonism: e.g., the μ-selective agonist dermorphin was converted to a δ selective antagonist [25]. In the search for other δ-antagonists, Schiller et al. [26,27] described a novel series of Tic^2-containing pseudopeptides, TIP(P) and TIP(P)[ψ], which displayed high δ-opioid affinity and selectivity. However, in the bioactive di- and tripeptide analogues, the Tyr-Tic moiety was recognized as the message domain and related to the rigid shape of a nonpeptide δ-opiate antagonist [28]. Augmentation of δ-selectivity and antagonism in these message domain peptides by the replacement of Tyr^1 with the more hydrophobic Dmt yielded ultraselective δ-opioid di- and tripeptide antagonists [29]. Further enhancement of the potency was attained by increasing the hydrophobicity of Dmt-Tic-OH by *N,N*-dialkylation with methyl groups to form an N-terminal tertiary amine [30]. On the other hand, incorporation of Dmt into DIPP yielded a moderately selective antagonist [31], while other Dmt-containing analogues exhibited different properties: e.g., [Dmt^1]DPDPE exhibited weak selectivity [32] or nonselectivity [33]; Dmt-D-Ala-arylalkylamide was moderately μ-selective [34]; and [Dmt^1]deltorphin B completely lost selectivity through the acquisition of dual high affinity for both μ and δ-receptors [35].

Therefore, considering the marked changes observed with the activity of δ- and μ-opioid peptides by the incorporation of Dmt and Tic residues we sought to determine whether similar substitutions in the N-terminal region of DYN could alter their κ-receptor agonist characteristics and convert the resulting peptide into an antagonist. We report herein the synthesis and structure–activity relationships among [Tic^2]DYN analogues (**1–3**) and a chimeric compound containing the N-terminal Tyr-D-Ala-Phe message domain of the amphibian opioid peptides [18] and the C-terminal octapeptide sequence of DYN A-(1-11)-NH_2 (**4**).

2. Results and discussion

Substitution of Tic for Gly^2 in DYN A-(1-13)-NH_2 (**1**) or -(1-11)-NH_2 (**2**) greatly reduced the affinity for κ- and μ-receptors, while only marginally effecting binding to δ-receptors (Table 1). The bisubstitution of Dmt^1-Tic^2 (**3**) dramatically increased δ-affinity by nearly two orders of magnitude, comparable to the δ-affinity observed with deltorphins [18–23]. On the other hand, μ-affinity of **3** rose 20-fold relative to **1** and **2**, while κ-affinity increased over 40-fold in guinea-pig brain membranes, ca. 20-fold in rat brain synaptosomes compared to **2**, and approximately 40-fold for both membrane preparations relative to **1**. The behavior of **3** resembles some Dmt^1-containing opioids where higher δ- and μ-affinities were recorded; e.g., [Dmt^1]-containing opioid heptapeptides had enhanced agonist activities [32,35] and the Dmt-Tic di- and tripeptides attained extraordinary antagonist activity [29]. Inclusion of the Tyr-D-Ala-Phe message domain (**4**) essentially eliminated opioid interaction with κ-receptors, whereas δ- and

Table 1
Receptor binding data for **1–4** and related peptides

Peptide	K_i values (nM)		κ-Receptor binding (K_i, nM)	
	δ	μ	Rat	Guinea pig
1. [Tic^2]DYN-A-(1-13)-NH_2	24.3 ± 8.9 (4)	85.4 ± 17.2 (5)	178.1 ± 36.6 (4)	118.0 ± 11 (4)
2. [Tic^2]DYN-A-(1-11)-NH_2	30.0 ± 8.4 (4)	91.7 ± 17.8 (5)	91.7 ± 18.9 (4)	145.6 ± 19 (4)
3. [Dmt^1,Tic^2]DYN-A-(1-11)-NH_2	0.39 ± 0.07 (4)	4.15 ± 1.3 (4)	4.29 ± 0.97 (4)	3.32 ± 0.49 (4)
4. [D-Ala^2,des-Gly^3]DYN-A-(1-11)-NH_2	39.9 ± 10.3 (4)	39.2 ± 11.0 (5)	22,450 ± 4,640 (3)	1,435 ± 106 (4)
DYN A-(1-11)-NH_2[a]	10.66 ± 0.67	2.71 ± 0.96	—	0.128 ± 0.038
Tyr-Tic-NH_2[b]	166 ± 48 (4)	28,712 ± 3,305 (7)	—	—
[Tic^2,Leu^5]enkephalin[c]	94 ± 21 (4)	4,620 ± 529 (3)	—	—
[Tic^2]dermorphin[c]	2.4 ± 0.7 (4)	204 ± 42 (4)	—	—

The numbers in parentheses are the number of independent repetitions (*n* value) with different synaptosomal preparations.
[a]Data from Ref. [48].
[b][28]. The K_i for κ-binding of Tyr-Tic-OH to rat synaptosomes was > 60 μM [23].
[c][25].

μ-affinities were only moderately altered in comparison to DYN A-(1-11)-NH_2 (Table 1). In the RJ bioassay, **1–3** antagonized the potent δ_{d2}-opioid receptor agonist deltorphin B with a pA_2 ranging from 7.25 to 8.12. The agonist activity of **4** on GPI was due only to the interaction with μ-receptors rather than κ-receptors because **1–3**, which are κ-antagonists, failed to modify the IC_{50} of **4** in the same tissue (data not shown).

Analogues **1–3** exhibited antagonist activity on GPI (μ- and κ-receptors) and RJ (δ-receptors), while **4** was a full agonist on both tissues (Table 2). In the GPI assay, peptides **1–3** antagonized the κ-agonist U-50,488 with weaker activity than **2**, which is a potent antagonist ($pA_2 = 8.33$). Although an apparent discrepancy occurred between **2** and **3** in receptor binding (Table 1) and functional bioassays (Table 2), the data for each peptide are consistent for a given assay (i.e., **2** weakly interacted with δ-, μ-, and κ-opioid receptors while the opposite was true for **3**). The anomalous behavior noted previously by other investigators [23,36–38] between receptor affinity and bioassays may be due to several factors. One very likely possibility is alternative splicing in κ-receptor mRNA [39] or alternate transcription start sites [40] that could produce multiple κ-receptor variants in a tissue-specific manner and account for the differential recognition of pharmacologically defined receptor subtypes. Other modifications related to receptor conformation that should be considered include changes initiated by interaction of G-coupled proteins with the intracellular loops and differences in phosphorylation of the C-terminal region which would affect the geometry of the receptor ligand-binding domain to differentially affect opioid receptivity [41]. Furthermore, extracellular cations could also affect both peptide and receptor structures to cause a differential shift in the observed response between tissue and isolated membrane receptors. Another suggestion that the overall 3-D shape and charge distribution of the peptide ligand could dictate binding [42] was postulated by Crescenzi et al. [43]. They proposed that the discrepancy in the receptor binding and bioactivity is directly related to the shape of the peptide ligand in solution since the 'combined effects in entropic gain' and the amounts of rotamer 3g- relative to 3t have approximately the same orders of magnitude in difference as observed between δ-receptor binding and bioactivity.

Although a shift in receptor activity toward δ-selectivity was reported in proceedings reports by Schmidt *et al.* [44] with [Tic^2,Phe^{3,4},D-Pro^{10}]DYN A-(1-11)-NH_2 and by Kulkarni et al. [45] with [Tic^2,Phe^{3,4},D-Pro^{10}]- and [Tic^2, D-Pro^{11}]DYN A-(1-11)-NH_2; only the former note presented limited pharmacological data. Therefore, a comparative interpretation of the data between those analogues and the data on the peptides reported herein **1–4** (Tables 1 and 2) may lead to erroneous interpretations.

3. Conclusion

Our results further demonstrated that Tic replacement of Gly^2 in DYN A analogues yielded potent δ- (**2**) and κ-opioid antagonism (**3**), while **2** exhibited the highest binding affinities to δ, μ, and κ receptors. These findings further support the observations that Tic^2 substitutions in dermorphin (a μ-selective agonist [25,44]) and [Leu^5]enkephalin (a δ-agonist [25]) produced peptides with δ-antagonism, suggesting that Tic in the second position of opioid agonists might represent a key factor in eliciting antagonism. However, Schmidt et al. [44] reported that [Tic^2]deltorphin C retained partial agonist activity in vitro in the MVD bioassay, whereas a deltorphin C octapeptide analogue, [Tic^2, Phe^{3,4}, Asp^5, Val^{6,7}, Gly^8-NH_2], was an antagonist with more δ-receptor selective properties. Thus, the overwhelming

Table 2
Functional bioassay data for **1–4** and related peptides

Peptide	Guinea pig ileum			Rabbit jejunum		
	pA_2	K_e, nM	IC_{50}, nM	pA_2	K_e, nM	IC_{50}, μM
1. [Tic^2]DYN-A-(1-13)-NH_2	6.34 ± 0.9	457	—	7.35 ± 0.7	44.7	—
2. [Tic^2]DYN-A-(1-11)-NH_2	8.33 ± 0.5	4.68	—	7.63 ± 0.9	23.4	—
3. [Dmt^1,Tic^2]DYN-A-(1-11)-NH_2	7.04 ± 0.7	91.2	—	8.12 ± 1.3	7.59	—
4. [D-Ala^2,des-Gly^3]DYN-A-(1-11)-NH_2	—	77	—	—	340	—
DYN A-(1-11)-NH_2[a]	—	—	2.88 ± 0.28	—	—	13.09 ± 2.15
Tyr-Tic-NH_2[b]	—	—	> 10,000	6.0	—	—
[Tic^2,Leu^5]enkephalin[c]	—	—	> 10,000	6.5	—	—
[Tic^2]dermorphin[c]	—	—	> 10,000	7.5	—	—

[a]Data from Ref. [48].
[b][28].
[c][25].
[d]Antagonism of U-50,488 (κ-selective agonist) induced inhibition of electrically induced contractions of GPI.
Peptides **1–3** are not antagonists on GPI using DAGO (μ-selective agonist) at a concentration > 10 μM.

preponderance of data confirm and substantiate that Tic in the second position of opioid peptides produces compounds with δ antagonism [25–29,31].

4. Experimental

4.1 Peptide synthesis

All peptides reported in this work were prepared by solid-phase synthesis using a Milligen 9050 synthesizer using 4-(2′,4′-dimethoxyphenol-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin (Rink amide MBHA resin; 0.55 mmol/g; 0.2 g) mixed with glass beads (1:15, w/w). Peptides were assembled using Fmoc-protected amino acids and 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole as coupling agents (1 h at each coupling step). Double coupling was required in the Lys-Leu, Arg-Pro, Phe-Leu, and Tyr- or Dmt-Tic acylation step. Side-chain protecting groups used were Pmc for Arg, Boc for Lys, and *t*Bu for Tyr. Deprotection and cleavage from the resin were accomplished with 88% TFA in the presence of 7% ET_3SiH and 5% H_2O (10 ml/0.2 g resin) for 1 h at room temperature.

4.2 Peptide purification

Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta prep 4000 system with a Waters PrepLC column (C18, 30×4 cm, 300 Å, 15 μm spherical particle size). The peptides were eluted with a gradient of 0–50% B in 25 min using mobile phases A (10% acetonitrile in 0.1% TFA, v/v) and B (60% acetonitrile in 0.1% TFA, v/v) at a flow rate of 50 ml/min.

Analytical HPLC was performed on a Bruker liquid chromatography LC21-C instrument fitted with a Vydac C18 column (4.6×250 mm, 5 μm particle size) with the above solvent systems with a linear gradient from 0–40% B in 25 min (I) or from 0–25% B in 25 min (II) at a flow rate of 1 ml/min. All analogues showed less than 1% impurities when monitored at 220 nm. Analytical determination of the capacity factor (K′) was determined using HPLC conditions in the above systems programmed at a flow rate of 1 ml/min (Table 3).

Amino acid analyses used Pico-Tag methodology and PITC amino acid derivatization. Lyophilysed samples of peptides (50–1,000 pmol) were placed in heat-treated borosilicate tubes (50×4 mm), sealed and hydrolysed in the Pico-Tag workstation with 200 ml 6 N HCl containing 1% phenol for 1 h at 150°C. A Pico-Tag column (15×3.9 mm) was employed to separate the PITC-amino acid derivatives. Final product confirmation was obtained by a MALDI-TOF analysis using a Hewlett–Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix. In all cases, the anticipated molecular weights were confirmed by MALDI-TOF. Table 3 lists the analytical properties of the analogues.

4.3 Receptor assays

Receptor binding assays were conducted according to published protocols [23,29,35] using 6.0 nM [^{3}H]DPDPE (32.0 Ci/mmol, NEN-DuPont) for δ-receptors, 3.5 nM [^{3}H]DAGO (60.0 Ci/mol, Amersham) for μ-receptors and 1.6 nM [^{3}H]bremazocine (1.0 m Ci/mmol, NEN-DuPont) for κ-receptors. Briefly, the assays contained 50 mM HEPES, pH 7.5, protease inhibitors, BSA, and $MgCl_2$ (1, 5, and 10 mM for μ-, δ-, and κ-receptors, respectively), and the κ-assay included 200 nM each DPDPE and DAGO to suppress binding to δ- and μ-sites. Brain synaptosome preparations (P_2 fraction) were preincubated and extensively washed to remove endogenous ligands, and stored in 20% glycerol at −80°C [35]. The δ- and μ-assays were incubated at room temperature (22–23°C) for 120 min, while the κ-assay was kept in the dark at 4°C for 40 min, before rapid filtration through GF/C filters. The K_i values were determined according to Cheng and Prusoff [46].

4.4 Bioassays

Guinea-pig ileum (GPI) and rabbit jejunum (RJ) were prepared as described previously [29]. Pieces of GPI

Table 3
Analytical properties of **1–4**

Compound	K′ values[a]			Amino Acid analyses[b]								
	I	II	MH^+	Y	A	G	F	L	R	I	P	K
1	5.27	6.03	1706	1.02	—	0.98	1	1.93	2.87	0.92	1.01	2.08
2	6.48	6.82	1464	0.97	—	1.02	1	1.03	3.02	0.95	0.97	1.04
3	7.21	7.76	1492	—	—	0.96	1	0.97	2.91	1.01	0.92	1.07
4	4.52	5.04	1319	1.05	0.98	—	1	1.07	2.94	1.03	0.98	1.03

[a]I and II refers to the HPLC gradients described in Experimental.
[b]Dmt and Tic residues were not quantitatively determined as PITC derivatives. Amino acid analyses are relative to F (phenylalanine). Other single letter codes define the following amino acids: Y, tyrosine; A, alanine; G, glycine; L, leucine; R, arginine; I, isoleucine; P, proline; K, lysine.

(2–3 cm) were incubated in 10 ml organ bath containing Tyrode's solution with 5% CO_2 in 95% oxygen at 37°C. A single RJ was dissected and suspended in 4 ml modified Krebs' solution aerated with the same gas mixture at 33°C. The twitches were recorded by means of an isometric transducer. Each analogue was tested for its ability to inhibit electrically evoked contractions (agonist activity) as well as the ability to antagonize the inhibitory effects of U50-488 (κ-selective agonist) and DAGO (μ-selective agonist) on GPI and deltorphin C (δ_1-receptor agonist) in the RJ bioassay. The IC_{50} value is the concentration of peptide required to inhibit these twitches by 50%. Multiple agonist dose response curves were obtained in the absence and in the presence of multiple concentrations of antagonist compounds for the calculation of pA_2 values [47].

Acknowledgements

This work was supported in part by MURST to S. Salvadori. We thank F. T. Lyndon at the NIEHS for expedient library services and the constructive comments of the ad hoc reviewers.

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[14] Symbols and abbreviations used in this paper are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (J Biol Chem 1972;247: 977). Other abbreviations: DYN, dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln); MVD, mouse vas deferens; GPI, guinea-pig ileum; RJ, rabbit jejunum; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP(P), Tyr-Tic-Phe-(Phe)-OH; TIP(P)[ψ], Tyr-Tic-ψ(CH_2-NH)-Phe-(Phe)-OH; Dmt, 2′,6′-dimethyl-L-tyrosine; DIPP, Dmt-Tic-Phe-Phe-OH; DPDPE, cyclo[D-$Pen^{2,5}$]enkephalin; Boc, tert-butyloxycarbonyl; Fmoc, (fluoren-9-ylmethoxy)carbonyl; Rink resin, 4-(2′,4′-dimethoxyphenyl)-Fmoc-aminomethylphenoxy-resin; Pmc, 2,2,5, 7,8-pentamethyl-chroman-6-sulfonyl; *t*Bu, *t*-butyl; TFA, trifluoroacetic acid; HPLC; high performance liquid chromatography; ET_3SiH, triethylsilane; MBHA, methyl-benzhydrylamine; DAGO, [D-Ala^2,NMe-Phe^4, Gly-ol]enkephalin; PITC, phenyl isothiocyanate; MH^+, molecular mass ion; pA_2, negative log of the molar concentration of antagonist required to double the concentration of agonist needed to elicit the original response; K_e, the antilog of pA_2; deltorphin C, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, deltorphin B, [Glu^4]deltorphin C; K_i, inhibition constant.
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