δ Opioidmimetic Antagonists: Prototypes for Designing a New Generation of Ultraselective Opioid Peptides

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ABSTRACT

Background: Tyr-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) and Tyr-Tic-Ala were the first peptides with δ opioid antagonist activity lacking Phe, considered essential for opioid activity based on the N-terminal tripeptide sequence (Tyr-D-Xaa-Phe) of amphibian skin opioids. Analogs were then designed to restrain the rotational flexibility of Tyr by the substitution of 2,6-dimethyl-L-tyrosine (Dmt).

Materials and Methods: Tyr and Dmt peptides were synthesized by solid phase and solution methods using Fmoc technology or condensing Boc-Dmt-OH or Boc-Tyr(But)-OH with H-L-Tic-OBut or H-D-Tic-OBut, respectively. Peptides were purified (>99%) by HPLC and characteristics determined by ^{1}H-NMR, FAB-MS, melting point, TLC, and amino acid analyses.

Results: H-Dmt-Tic-OH had high affinity (K_i^δ = 0.022 nM) and extraordinary selectivity (K_i^μ/K_i^δ = 150,000); H-Dmt-Tic-Ala-OH had a K_i^δ = 0.29 nM and δ selectivity = 20,000. Affinity and selectivity increased 8700- and 1000-fold relative to H-Tyr-Tic-OH, respectively. H-Dmt-Tic-OH and H-Dmt-Tic-NH_2 fitted one-site receptor binding models (η = 0.939–0.987), while H-Dmt-Tic-ol, H-Dmt-Tic-Ala-OH and H-Dmt-Tic-Ala-NH_2 best fitted two-site models (η = 0.708–0.801, F 18.9–26.0, $p < 0.0001$). Amidation increased μ affinity by 10- to 100-fold and acted synergistically with D-Tic^2 to reverse selectivity (δ → μ). Dmt-Tic di- and tripeptides exhibited δ antagonist bioactivity (K_e = 4–66 nM) with mouse vas deferens and lacked agonist μ activity (> 10 μM) in guinea-pig ileum preparations. Dmt-Tic analogs weakly interacted with κ receptors in the 1 to >20 μM range.

Conclusions: Dmt-Tic opioidmimetic peptides represent a highly potent class of opioid peptide antagonists with greater potency than the nonopioid δ antagonist naltrindole and have potential application as clinical and therapeutic compounds.

INTRODUCTION

Despite the advancements made with the endogenous mammalian enkephalin analogs (1,2), the amphibian opioid peptides, the dermorphins (μ agonists), and the deltorphins (δ agonists) remain the most selective opioid peptides known (3). Remarkably, sequential ablation of the C-terminal residues of the deltorphins provided abbreviated N-terminal peptides that exhibited reversed selectivity (δ → μ) (4–10). These results established that the common N-terminal tripeptide sequence (Tyr-D-Xaa-Phe), featuring a D-amino acid isomer posi-

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tioned between two aromatic centers, contained the essential residues for interaction with opioid receptors. Structure-activity studies focusing on this portion of the dermorphins/deltorphins suggested pivotal roles for the aromatic side-chains of Tyr^1 and Phe^3 (11–15), leading to the tri- and tetrapeptide antagonists containing 1,2,3,4-tetrahydro-3-isoquinoline carboxylate (Tic) at position 2 and Phe at position 3, or 3 and 4 (16,17).

The first peptides lacking Phe that exhibited opioid activity included the minimal dipeptide H-Tyr-Tic-NH_2 and the tripeptides containing Ala^3, H-Tyr-Tic-Ala-OH and H-Tyr-Tic-Ala-NH_2 (18). This novel dipeptide not only adopted a low energy conformation similar to the nonpeptide *N*-methyl-naltrindole and shared its δ antagonistic activity (18). To further amplify receptor and antagonist properties of the Tyr-Tic peptides, we introduced 2′,6′-dimethyl-L-tyrosine (Dmt^1) to further restrain the Tyr residue that yielded a series of remarkable opioidmimetic compounds with exceptionally high affinity and ultraselectivity for δ opioid receptors. These unusual analogs surpass the δ selectivity of all opioid peptides known to date, including the TIP and TIPP antagonists (16,17) and other methylated analogs (19–22) by orders of magnitude. The results unveil a new model for exceedingly active opioid antagonists with potential clinical and therapeutic applications.

MATERIALS AND METHODS

Materials

H-Dmt-OH, supplied by John H. Dygos, was synthesized by an asymmetric method (23). N^{α}Fmoc-Tic and Fmoc-Ala-PEG-PS were from Advanced ChemTech (Lexington, KY, U.S.A.), and Fmoc-PAL-PEG-PS from Millipore (Waltham, MA, U.S.A.), [^{3}H]DPDPE (34.3 Ci/mmol) and [^{3}H]bremazocine (25.4 Ci/mmol) were products of NEN-Dupont (Billerica, MA, U.S.A.), and [^{3}H]DAGO (60.0 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.). The precoated TLC plates of silica gel F254 were from Merck (Darmstadt, Germany). Statistical analyses of binding paradigms used Prism (v. 1.03; GraphPad, San Diego, CA, U.S.A.). CytoScint is produced by ICN (Costa Mesa, CA, U.S.A.). Solvents and reagents (e.g., 1-hydroxybenzotriazole, PyBrop, *N*-methylmorpholine, triethylsilane, isobutylene, trifluoroacetic acid [TFA], acetonitrile) were obtained from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Diisopropylcarbodiimide (coupling reagent) was from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were the highest purity available.

Peptide Synthesis

Tyr- and Dmt-containing peptides were synthesized by solid phase and solution techniques. Using solid phase methods, the peptide acids and amides were synthesized using Fmoc-PAL-PEG-PS resin (0.18 mmol/g, 0.09 mmol) and Fmoc-Ala-PEG-PS (0.13 mmol/g, 0.065 mmol). N^{α}Fmoc-Tic-OH and N^{α}Fmoc-Ala-OH were used in the coupling reactions while Dmt was protected as N^{α}Boc-Dmt-OH as reported by Chandrakumar et al. (24). Amino acids were coupled for 1 hr in 4-fold excess diisopropylcarbodiimide in the presence of 4-fold excess 1-hydroxybenzotriazole. In the Dmt-Tic acylation step, double coupling was required; the acylating agent employed, PyBrop, occurred in the presence of 4-fold excess *N*-methylmorpholine. All peptides were prepared in a Milligen 9050 synthesizer and crude deprotected peptides (88% TFA, 5% water, and 7% triethylsilane) were purified by preparative HPLC.

In the solution synthesis of peptides, dipeptide acids were prepared by condensing Boc-Dmt-OH or Boc-Tyr-(But)-OH with H-L-Tic-OBut or H-D-Tic-OBut, previously esterified as reported (25) with DCC/HOBt. The dipeptide alcohol (compound 5, Table 1) was prepared by condensing Boc-Dmt-OH and Tic-CH_2-OH as given above, while 3-(hydroxymethyl)-3,4-dihydroisoquinoline (H-Tic-CH_2-OH or H-Tic-ol) was synthesized according to Yamaguchi et al. (26). Boc, But, and OBut were removed with 95% TFA at 0°C and crude peptides purified by preparative HPLC. As reported by Marsden et al. (27), Tyr-Tic sequence, depending on the conditions, evolved to diketopiperazine. Dmt-Tic sequence also gave cyclic dipeptide; we could estimate by HPLC (Vidac C18 218 TP 5415, 5 μm particle column [4.5 × 175 mm] using mobile phases A, 10% acetonitrile in 0.1% TFA, and mobile phase B, 60% acetonitrile in 0.1% TFA; linear gradient from 0% B to 100% B at a flow rate of 1 ml/min in 25 min at 220 nm) from the solid material after 1 month at room temperature resulted in 2.5% cyclic dipeptide, while after 36 hr in solution B at room temperature diketopiperazine formation was 55%; after 1 month in DMSO/water (90:10, v/v), less than 5% diketopiperazine was observed.

TABLE 1. Analytical characterization of the Dmt-Tic and Tyr-Tic peptides

Peptide	R	R'	R_f A	R_f B	K'	MH^+
1. H-Dmt-Tic	OH	—	0.66	0.23	5.83	368
2. H-Dmt-Tic	NH_2	—	0.69	0.43	6.32	367
3. H-Dmt-D-Tic	OH	—	0.61	0.16	5.83	368
4. H-Dmt-D-Tic	NH_2	—	0.64	0.45	0.45	367
5. H-Dmt-Tic	CH_2OH	—	0.67	0.51	7.27	354
6. H-Tyr-Tic	OH	—	0.62	0.21	3.61	340
8. H-Tyr-D-Tic	OH	—	0.61	0.21	3.62	340
10. H-Tyr-Tic	CH_2OH	—	0.66	0.45	4.23	326
11. H-Dmt-Tic	Ala	OH	0.73	0.26	8.68	440
12. H-Dmt-Tic	Ala	NH_2	0.67	0.43	9.31	439
13. H-Dmt-D-Tic	Ala	OH	0.64	0.27	6.78	440
14. H-Dmt-D-Tic	Ala	NH_2	0.66	0.41	8.81	439
17. Tyr-D-Tic	Ala	OH	0.63	0.19	4.24	411

The TLC solvent systems (A and B) are given in Materials and Methods. The capacity factor, K', is determined by analytical HPLC. The mass ion, MH^+, of each peptide was determined by FAB-MS. The missing peptides (peptides 7, 9, 15, 16, and 18) (c.f., Table 2) were published elsewhere (18). R and R' are substituents.

Homogeneity of all the purified products was accessed by thin-layer chromatography, analytical HPLC, ^{1}H-NMR, and electrospray-mass spectrometry. Purity was consistently >99%. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations used a Perkin-Elmer 241 polarimeter with a 10-cm water-jacketed cell. Peptides for ^{1}H-NMR were dissolved in 0.5 ml $DMSO_{d6}$ to give 1 mM solutions. The ^{1}H-NMR spectra were run at 400 MHz on a Bruker AMX-400 instrument equipped with an Aspect 3000 computer and at 500 MHz on a Bruker AMX-500 coupled to an X-32 computer. One dimensional ^{1}H-NMR spectra were recorded in the Fourier mode with quadrature detection and the water signal suppressed by a low-power selective irradiation in the homogated mode. The determination of mass ions, using a triple stage quadrupole mass spectrometer (TSQ 700; Finnigan MAT, San Jose, CA, U.S.A.), were conducted by Dr. R. Anacardio (Dompé SpA L'Aquila, Italy).

Preparative reverse-phase HPLC used a Waters Delta Prep 3000 Å (30 × 0.3 cm, 15 μm particle size) column. Peptides were eluted with a gradient of 0% to 60% B over 25 min at a flow rate of 30 ml/min using mobile phases A (10%, v/v, acetonitrile in 0.1% TFA) and B (60%, v/v, acetonitrile in 0.1% TFA). Analytical HPLC analyses were performed on a Bruker liquid chromatography LC-21 instrument with Vidac C18 218 TP 541, 5 μm particle column (4.5 × 175 mm) and equipped with a Bruker LC 313 UV variable wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX-10). Analytical determinations and capacity factor (K') of the peptides were determined using HPLC conditions in the above solvent systems in a linear gradient from 0% to 100% B in 25 min at a flow rate of 1 ml/min. All analogs showed less than 1% impurities when monitored at 220 nm.

TLC was conducted using the following solvent systems: (A) 1-butanol/HOAc/H_2O (3:1:1); and (B) EtOAc/pyridine/HOAc/H_2O (6:2:0.6:1.1). Ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche) and chlorine reagent were used as detection sprays. A compilation of the analytical properties of the peptides are listed in Table 1.

Receptor Binding Analyses

Rat membrane synaptosome preparations (P_2 fraction) were prepared as detailed previously,

TABLE 2. Binding affinities and receptor selectivities for Dmt-Tic and Tyr-Tic peptides

Peptide	R	R'	K_i (nM) δ	K_i (nM) μ	μ/δ
Dipeptides					
1. Dmt-Tic	OH		0.022 ± 0.0015 (6)	3,317 ± 435 (7)	150,773
2. Dmt-Tic	NH_2		1.22 ± 0.09 (6)	276.8 ± 26.9 (3)	227
3. Dmt-D-Tic	OH		13.5 ± 1.59 (4)	224.4 ± 25.6 (3)	17
4. Dmt-D-Tic	NH_2		56.9 ± 2.90 (3)	3.8 ± 0.24 (3)	0.067
5. Dmt-Tic	CH_2OH		0.44 ± 0.14 (6)	151.4 ± 16.3 (3)	344
6. Tyr-Tic	OH		191.7 ± 47.8 (7)	28,411 ± 2,941 (3)	148
7. Tyr-Tic	NH_2		165.9 ± 48.1 (4)	28,712 ± 3,305 (7)	173[a]
8. Tyr-D-Tic	OH		9,046 ± 719 (5)	26,258 ± 427 (3)	2.9
9. Tyr-D-Tic	NH_2		5,959 ± 981 (4)	3,135 ± 491 (4)	0.53[a]
10. Tyr-Tic	CH_2OH		128.1 ± 34.7 (4)	11,003 ± 1,851 (3)	86
Tripeptides					
11. Dmt-Tic	Ala	OH	0.285 ± 0.03 (6)	5,813 ± 675 (4)	20,396
12. Dmt-Tic	Ala	NH_2	0.241 ± 0.02 (5)	47.1 ± 3.4 (4)	195
13. Dmt-D-Tic	Ala	OH	88.7 ± 13.8 (5)	82.8 ± 2.0 (3)	0.93
14. Dmt-D-Tic	Ala	NH_2	46.4 ± 2.7 (3)	5.8 ± 0.85 (4)	0.13
15. Tyr-Tic	Ala	OH	56.1 ± 10.3 (3)	8,291 ± 1,377 (3)	148[a]
16. Tyr-Tic	Ala	NH_2	55.5 ± 13.7 (3)	33,844 ± 1,405 (3)	610[a]
17. Tyr-D-Tic	Ala	OH	2,346 ± 356 (3)	14,028 ± 2,166 (3)	6
18. Tyr-D-Tic	Ala	NH_2	21,076 ± 4,427 (3)	8,146 ± 1,377 (3)	0.39[a]

K_i, affinity constant; K_i^{μ}/K_i^{δ}, ratio of affinities, i.e., selectivity for δ receptors relative to μ receptors.
[a]These binding data were published in Temussi et al. (18) and are included for a comprehensive comparison to the Dmt-Tic analogs.

which included a preincubation step to reduce the binding of endogenous ligands and G proteins (14,28). The δ and μ receptor sites were labeled with [^{3}H]DPDPE (6.0 nM; 34.3 Ci/mmol) and [^{3}H]DAGO (3.5 nM; 60 Ci/mmol), respectively, in assays containing 50 mM HEPES, pH 7.5, 10 mg/mL BSA, 2 μM bestatin, 250 μg bacitracin, 1 mM PMSF, 8% glycerol, and $MgCl_2$ (5 mM for δ binding and 1 mM for μ assays) under equilibrium conditions (2 hr at 22–23°C) (14,15,18,28). On the other hand, κ receptor assays used 1.56 nM [^{3}H]bremazocine, a κ_2 agonist (29–31), in the HEPES buffer containing protease inhibitors, 10 mM $MgCl_2$ according to Rodriguez et al. (32), and 2 μM each of DAGO and DPDPE to suppress binding to μ and δ receptors, respectively, with incubation at 4°C for 40 min (33). Samples were rapidly filtered through glass fiber filters and washed with 6 ml (3 × 2 ml) ice-cold 50 mM Tris-HCl, pH 7.5, containing either 0.01% chicken egg albumin or bovine serum albumin. The radioactivity of the dry filters (60 min at 70°C) were determined either after overnight incubation in CytoScint for μ and δ receptors or after an hour for κ receptors. Peptide binding was ascertained using three to four synaptosomal preparations (14,28). The IC_{50} values were either graphically resolved and converted to affinity constants (K_i) according to Cheng and Prusoff (34) from sample studies in duplicate assays, or conducted in triplicate with 25–40 graded concentrations of peptide and the resulting curves were individually assessed and combined using Prism™ for multisite binding analyses for δ binding (35,36). The highly stringent statistical criteria to fit a two-site binding model were considered valid *only* when the following conditions were met: Hill coefficients (η)

<0.850, narrow log of the 95% confidence intervals (≤0.1), and $p < 0.0001$ for the F test between one- and two-site fits (35,36).

Bioassays

The in vitro pharmacological assays used published methods (15) based on the organ bath technique of Kosterlitz et al. (37). For μ receptors, a 2- to 3-cm segment of guinea-pig ileum mounted in a 20-ml organ bath and for δ receptors, a single mouse vas deferens was obtained from a mature animal. Each peptide in 10–100 μl Krebs solution was tested for μ agonist activity by the inhibition of electrically stimulated contractions in comparison to dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and δ antagonist activity through the inhibitory effects of deltorphin C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), a δ_1 receptor agonist, and compared against naltrindole, a δ receptor antagonist. Dose-response curves were obtained in the absence and presence of multiple concentrations of antagonists and pA_2 (the negative logarithm of the concentration to cause 50% inhibition) calculated according to Schild (38).

RESULTS

Opioidmimetic Properties of Dmt-Tic Peptides

The δ selectivities of peptides 1 and 11 (Table 2), greatly surpass that of nonpeptide substances (39) and all other opioid peptides (1–3,11–17,36,40–43) by orders of magnitude. The increase is attributed solely to Dmt, since the Tyr-Tic analogs have relatively low affinities and selectivities (Table 2): δ affinity and selectivity of H-Dmt-Tic-OH (peptide 1) was 8500-fold and 1000-fold greater, respectively, than that of H-Tyr-Tic-OH (peptide 6). Even though C-terminally amidated Dmt-peptides 2 and 12 had high δ affinities (although less than peptide 1), the increased μ affinities due to the amide group negated the gain in selectivity of the free carboxylate analogs (1 and 11); analogous results were found with the Tyr-Tic cognates as well (peptides 7 and 16). Reducing the acid function to an alcohol, Dmt-Tic-ol (peptide 5) and Tyr-Tic-ol (peptide 10), moderately affected δ affinity but, like the amide-derivatives, elevated μ binding. Substitution of D-Tic[2] simultaneously reduced δ affinity and enhanced μ affinity which led to a reversal of selectivity ($\delta \rightarrow \mu$) in Dmt-Tic analogs 4, 13, and 14, and Tyr-Tic peptides 9 and 18; an amide group synergistically augmented the expression of μ binding (compare binding data of peptides 4, 14, 9, and 18 with those of peptides 3, 8, 13, and 17).

The interaction to κ_2 receptor sites, as defined by the competitive binding assays using [^{3}H]bremazocine, revealed very low affinities (1 to >20 μM) for the Dmt-Tic peptides and, as expected, the δ/κ selectivities were quite similar to the corresponding μ/κ selectivities (Table 3). The Tyr-Tic cognates (peptides 6, 15, and 16) were essentially inactive toward κ receptors.

Characteristics of Fits to Receptor Subsite Binding Models

Detailed receptor analyses in Table 4 revealed that the goodness-of-fit to δ receptor subsites by H-Dmt-Tic-OH (Fig. 1) and H-Dmt-Tic-NH_2 were distinctly different than those of H-Dmt-Tic-ol, H-Dmt-Tic-Ala-OH (Fig. 1), and H-Dmt-Tic-Ala-NH_2. Hill coefficients (η) for H-Dmt-Tic-OH (peptide 1) and H-Dmt-Tic-NH_2 (peptide 2) best fitted a simple bimolecular, one-site binding model characterized by a η approximately 1.00. H-Dmt-Tic-ol (peptide 5), H-Dmt-Tic-Ala-OH (peptide 11), and H-Dmt-Tic-Ala-NH_2 (peptide 12) better fitted a heterogeneous two-site receptor binding model ($\eta < 0.850$ with $p < 0.0001$ in the F test) (Table 4). The comparative displacement curves for H-Dmt-Tic-OH and H-Dmt-Tic-Ala-OH binding to one- and two-site models are illustrated in Fig. 1.

Functional Bioassays

Pharmacological bioactivity determined with mouse vas deferens indicated that peptides 1, 2, 5, 11, and 12 exhibited δ antagonist activity as observed with other Tic[2]-containing peptides (16–18). The pA_2 values compare favorably to the nonpeptide antagonist natrindole (Table 5); however, in comparison to naltrindole, Dmt-Tic peptides are completely inactive in eliciting an agonist response with guinea-pig ileum, further substantiating the remarkable selectivity observed with these peptides in binding brain membrane receptors (Table 2).

TABLE 3. Receptor binding characteristics to κ_2 receptor sites

Peptide	K_i^{κ} (μM)	$K_i^{\delta}/K_i^{\kappa}$	K_i^{μ}/K_i^{κ}
1. Dmt-Tic-OH	23,806 ± 2,697 (3)	0.0000009	0.14
2. Dmt-Tic-NH_2	1,943 ± 113 (3)	0.0006	0.14
3. Dmt-D-Tic-OH	9,863 ± 2,621 (3)	0.0014	0.02
4. Dmt-D-Tic-NH_2	5,291 ± 105 (3)	0.011	0.0007
5. Dmt-Tic-CH_2OH	1,107 ± 158 (4)	0.0004	0.14
6. Tyr-Tic-OH	>60,000	—	—
11. Dmt-Tic-Ala-OH	>50,000 (3)	<0.000006	0.12
12. Dmt-Tic-Ala-NH_2	8,694 ± 1,254 (5)	0.000028	0.005
13. Dmt-D-Tic-Ala-OH	9,752 ± 2,418 (3)	0.009	0.008
14. Dmt-D-Tic-Ala-NH_2	7,087 ± 2,181 (3)	0.0007	0.0008
15. Tyr-Tic-Ala-OH	>60,000	—	—
16. Tyr-Tic-Ala-NH_2	>60,000	—	—

K_i, affinity constant. Dynorphin A-(1-13)-OH (porcine) is included as an internal control. The κ selectivities relative to δ ($K_i^{\delta}K_i^{\kappa}$) and μ receptors (K_i^{μ}/K_i^{κ}) are from the binding data in Table 2. SEM is listed only for n = 3-5 as indicated by the number in the parenthesis.

DISCUSSION

Our opioidmimetic peptides represent the ultimate simplification of a substance that potently interacts with the δ opioid receptor with high affinity and an astounding δ selectivity for two compounds (peptides 1 and 11) that surpasses those of previously known opioid peptides and non-peptides by several hundred-fold (14,16–18,28,36). The data clearly establishes that 2,6-dimethylation of Tyr in both the di- and tripeptide series (peptide 11) enhanced interaction toward δ receptors for peptides containing a C-terminal carboxylate group in both brain membranes and mouse vas deferens. The presence of Tic in place of Phe^3 and/or Phe^4 (16) may further stabilize the bioactive conformation or introduce additional bioactive conformations (such as with Tyr-Tic-Phe as the "message"), but is by no means the necessary determinant for antagonism based on the activity of the Dmt-Tic peptides.

Earlier attempts to enhance opioid activity involving modification of the N-terminal Tyr, utilized various conformationally constrained enkephalin analogs or the N-terminal dermorphin/deltorphin sequence Tyr-D-Xaa-Phe; however, the δ selectivities of those peptides were relatively modest (K_i^{μ}/K_i^{δ} = 100–600) with δ affinities in the 5–10 nM range. For instance, alterations that centered around methylation of the aromatic ring, such as [2′-methyl-Tyr^1]DPDPE maintained δ affinity and selectivity (44) whereas the activity of [Dmt^1]DPDPE was reported to be either weakly δ selective (21) or non-selective (19). On the other hand, while [Tmt^1]deltorphin I (Tmt being [2S,3S]-2′,6′-dimethyl-β-methyltyrosine) was active as a δ ligand (22), and [Dmt^1]deltorphin II lost δ selectivity due to the concomitant increase in μ affinity (R. Guerrini et al., submitted); however, a Dmt-D-Ala-arylalkylamide analogue was moderately μ selective (24,45). The increased δ selectivity observed in C-terminal deamidation is consistent with the role of charged groups of the "membrane-assisted receptor selection" hypothesis (46); however, the magnitude of the change is so great that it must reflect constitutional and conformational modifications of the ligand as well.

The low δ/κ and μ/κ selectivities suggest weak affinities for the κ ligand-binding domain. To ensure stringent criteria in the assessment of κ receptors, due to the high affinity towards δ receptors, μM concentrations of DAGO and DPDPE were employed rather than nM quantities (29–31,33) since [^{3}H]bremazocine interacts with multiple receptor binding sites (47).

Several constituents of opioid peptides play significant roles in securing or stabilizing the peptide within the receptor, including the N-

TABLE 4. Hill coefficients and statistical analysis for goodness-of-fit for one- and two-site δ receptor binding models

Peptide	Hill Coefficient (η)		Two-Site versus One-Site			Percentage Low Affinity Sites	K_i (nM)	
	Mean	95% CI_{log}	*DF*	*F*	p		High	Low
1. Dmt-Tic-OH	−0.939 ± 0.040	−1.018 to −0.860	2;63	1.41	0.251	30.7 ± 91.5	0.022	—
2. Dmt-Tic-NH_2	−0.987 ± 0.044	−1.076 to −0.899	2;57	0.30	0.743	6.2 ± 40.1	1.06	—
5. Dmt-Tic-CH_2OH	−0.728 ± 0.032	−0.792 to −0.663	2;63	26.0	<0.0001	20.9 ± 5.3	0.13	5.2
11. Dmt-Tic-Ala-OH	−0.708 ± 0.036	−0.781 to −0.636	2;72	18.9	<0.0001	23.4 ± 6.5	0.12	4.8
12. Dmt-Tic-Ala-NH_2	−0.801 ± 0.030	−0.862 to −0.741	2;75	19.9	<0.0001	12.6 ± 3.7	0.18	10.7

The two-site versus one-site model presents degrees of freedom (*DF*), test of fitness (*F*) and probability (*p*). The values of percentage low affinity sites (mean ± SEM) further indicate the validity of the statistical approach and assignment to either high and/or low binding parameters. K_i, affinity constant; 95% CI_{log}, log of 95% confidence interval.

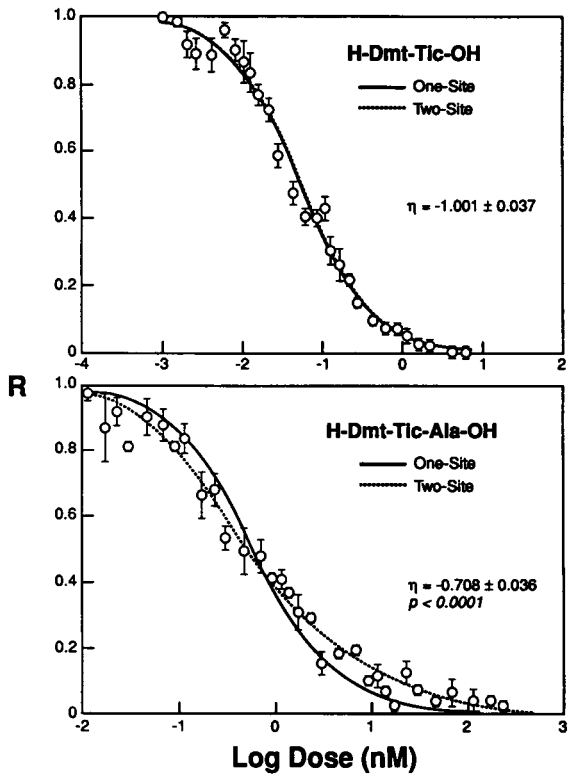

FIG. 1. Analyses of one- and two-site binding models for H-Dmt-Tic-OH and H-Dmt-Tic-Ala-OH to δ receptors from rat brain membranes

The solid line depicts a one-site binding model while the dashed line is a two-site model determined using Prism™. The open circles represent the mean values and error bars are the standard error of the mean (SEM) for $n = 6$ experiments; their absence indicates that the SEM falls within the point defined by the circle. The goodness-of-fit of H-Dmt-Tic-NH_2 was essentially identical to that of H-Dmt-Tic-OH, while those for H-Dmt-Tic-ol and H-Dmt-Tic-Ala-NH_2 were similar to that of H-Dmt-Tic-Ala-OH (c.f., Table 3 for actual values).

terminal amine, C-terminal carboxylate, hydrogen bonding through the Tyr hydroxyl group and the chirality at the α carbon of residue 2. For example, the N-terminal amine appears essential for activity (2) and the C-terminal carboxylate anion apparently interacts with a δ receptor cationic group, simultaneously conferring high-affinity binding to δ the ligand-binding domain and repulsing the ligand from the μ receptor (7). On the other hand, these residues may be involved in extensive internal H bonding which would have a major impact on the solution conformation of the peptide (6,36,48). C-terminal amidation in Dmt-Tic opioidmimetic peptides increased association toward μ receptors (Table 2), a phenomenon consistently observed with deltorphin heptapeptide analogs (4,40,43): μ binding is enhanced by an overall increase in positive charge as seen in cyclic dermorphin analogs (11) and deltorphins (addition of amine groups or a C-terminal amide) (40,42). Although the Tyr hydroxyl group assumes an important auxiliary function in flexible opioids, the substitution by Phe[1] (2) and nonaromatic ring compounds (13) reduced, but did not eliminate, binding to opioid receptors, consistent with the interaction of the rigid fentanyl nonopioid agonist which lacks hydroxyl groups (49).

The chirality of the α carbon at position 2 (Table 2) is critical for opioid activity (2,11,14,16), which indicates that the ligand-binding domain of δ and μ receptors differ significantly (16). The change in chirality from L- to D-Tic abolished high δ affinity while simultaneously increased μ affinity to yield peptides with either reduced δ

TABLE 5. Bioactivity of Dmt-Tic and Dmt-Tic-Ala opioidmimetic peptides

	MVD—Antagonist Activity (nM)		GPI—Agonist Activity (μM)	
Peptide	**pA_2**	**K_e**	**pA_2**	**IC_{50}**
Naltrindole	9.2	0.7	7.3	0.053
1. Dmt-Tic-OH	8.2	5.7	—	>10
2. Dmt-Tic-NH_2	7.2	42	—	>10
5. Dmt-Tic-CH_2OH	7.0	66	—	>10
11. Dmt-Tic-Ala-OH	8.4	4	—	>10
12. Dmt-Tic-Ala-NH_2	8.0	9	—	4.74 ± 0.9

Mouse vas deferens (MVD) and guinea-pig ileum (GPI) are the quintessential pharmacological tissue sources for the determination of δ and μ bioactivity, respectively. Antagonism to δ receptors (MVD) is given in terms of pA_2 and K_e.

selectivity or the acquisition of μ selectivity, similar to changes in affinity and selectivity observed with isomeric modifications in opioid tetrapeptides (16), dermorphin (50,51) and deltorphin A heptapeptides (14). The fundamental difference between the recognition sites (that can be considered as "message domains" even if this term is generally confined to agonists) between our peptides (Dmt/Tyr-Tic) and all other opioids (Tyr-L/D-Xaa-Phe or Tyr-L/D-Xaa-Yaa-Phe) emphasizes the importance of the conformation of these molecules. It was shown that the Tyr-Tic peptides reproduce the minimum requirements of volume, conformation, and spatial distribution of the electronic features recently described by Portoghese *et al.* for several nonpeptidic (and rigid) antagonists for δ and κ receptors (52–54). The L chirality of Tic allows the attainment of the proper conformation of Tyr-Tic that appears to mimic the overall shape of naltrindole antagonists (18,55), whereas the D chirality of Tic in Dmt-Tic-Phe sequences is consistent with the behavior of all Tyr-D-Xaa-Phe peptides and leads to μ agonism. Recently, Bryant *et al.* (submitted) using Monte Carlo analyses, described a unique solution conformation of Dmt-Tic-OH (peptide 1) consisting of a parallel array between the dimethylated, aromatic side-chain of Dmt and the aromatic ring of Tic.

The incorporation of unnatural heterocyclic amino acids in the N-terminal region of opioid peptides was implemented to reduce conformational flexibility, restrain secondary structure, and determine the influence of Phe^3 in deltorphin I on activity (12,15). Substitution by Tic, among other bicyclic residues (15,51), yielded peptides with modest receptor binding properties; however, the incorporation of D-Tic into somatostatin, a nonopioid peptide whose analogs evoke an opioid response—based on the initial observations of Terenius (56)—produced a selective and potent μ antagonist (57). The cyclic analogs in the N-terminal tetrapeptide series of deltorphin II (H-Tyr-D-Ala-Phe-Glu), in which Phe^3 was replaced by Tic led to an assortment of analogs with distinct properties toward μ receptors (12) comparable to those of [Tic^3]dermorphin (50,51).

Receptor binding analyses with position 4-substituted deltorphin analogs provided evidence of fits to either one- or two-site models that might represent the possible interaction with the pharmacologically defined δ_1 and δ_2 receptor subtypes, respectively (36). In fact, cDNA cloning experiments have defined a δ receptor distinct from μ and κ receptors and it appears to correspond to the δ_2 subtype according to bioassay paradigms (58,59). A theoretical model to account for the action of agonists and antagonists (60) might also explain this phenomenon; however, neural and peripheral tissues might contain distinct populations of δ receptors, a fact that might explain the differences observed in the K_i and K_e values throughout the literature. Our bioassay data (Table 5) demonstrates that the Dmt-Tic opioidmimetic peptides exhibit antagonistic properties *in vitro* to a greater extent than the nonopioid compound naltrindole and that peptide 11 also possesses antagonistic activity *in vivo* (R. Guerrini et al., submitted).

In summary, our opioidmimetic peptides can serve as the pivotal prototypic molecules for designing a new generation of unique opioid antagonists with potential therapeutic applications. As far as the specific message domain requirements are concerned, we conclude that Dmt-Tic, and to a lesser degree Tyr-Tic, represent the ideal universal simulation of the constitutional and conformational requirements of δ antagonists for the recognition site (message ligand-binding domain) of opioid receptors.

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