Design of δ-opioid peptide antagonists for emerging drug applications

Lawrence H. Lazarus, Sharon D. Bryant, Peter S. Cooper, Remo Guerrini, Gianfranco Balboni and Severo Salvadori

The need for δ-receptor-selective opioid antagonists has led to their development based on structure–activity relationships of δ- and μ-opioid agonists. The unusual amino acid 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), found in a series of H-Tyr-Tic-Phe-(Phe)-OH peptides, is an essential feature of derivatives discussed in this article. Elimination of Phe yields the H-Tyr-Tic-OH dipeptide antagonists, while substitution of Tyr by 2′,6′-dimethyl-L-tyrosine (Dmt) gives H-Dmt-Tic-OH and numerous potent, high-affinity and ultraselective δ-opioid antagonists. This article reviews the emergence of derivatives based on the Tyr-Tic and Dmt-Tic pharmacophores as lead structures, and discusses potential clinical and therapeutic applications.

The involvement of opiate alkaloids in the treatment of a diverse range of medical syndromes is found throughout human history, from the cuneiform tablets of the Assyrians to modern medical journals and the pages of our daily tabloids. Alkaloid opiates and their synthetic derivatives are currently used as agonists to treat acute and chronic pain, while antagonists (such as naltrindole, naltrexone and naloxone) are used not only to study the interaction of agonists, but also to assist patients fighting narcotic addiction. Furthermore, opiate antagonists find clinical application in relieving alcohol dependency[1], blocking the reinforcing properties of cocaine[2], modulating the behavioural effects of amphetamines[3], immunosuppression[4] during organ transplantation[5], treating autism[6] and Tourette's syndrome[7]. Even though the alkaloid derivatives have high affinity for one receptor type, they lack the high degree of selectivity required to pinpoint precisely which of the three known opioid receptor types (μ, δ or κ) is responsible for the biological effect being measured.

The discovery of the endogenous opioid peptides with their limited receptor selectivity more than two decades ago[8] implicated their potential involvement in analgesia and in a physiological context, including exercise (the 'no gain without pain' mantra). Unfortunately, the exogenous application of these opioid peptides and their analogues generally met with failure, owing to biological instability and inability to be transmitted through the blood–brain barrier unless structurally modified. From the structure–activity studies on many hundreds of enkephalin analogues[9], only two analogues of [Met^5]enkephalin have progressed to clinical trials (FK33824 and metkephamide)[10]. Extensive investigation of these two enkephalin analogues confirmed that the appropriate modification of a natural opioid peptide will produce a systemically active analgesic agent.

Opioid peptide agonists isolated from amphibian skin consist of two groups, the dermorphins (μ selective) and the deltorphins (δ selective)[11], which have enhanced biological stability[12,13] and are transported into the brain when

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administered *in vivo* and across blood vessels *in vitro*[14–16]. The hydrophobicity of a peptide is an important physicochemical property that enhances permeability through membrane barriers[14]. One means of enhancing the ability of a peptide to cross the blood–brain barrier is by substitution of selected residues with those of higher octanol-water coefficients[14], such as aliphatic, aromatic and heterocyclic amino acids. In deltorphins and dermorphins, the introduction of specific heterocyclic amino acids confers constraints on the peptide backbone and side-chain orientation. One prime example that led to the formation of δ-opioid antagonists was the substitution of the 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) residue in the second position of C-terminally abbreviated amphibian peptides to form H-Tyr-Tic-Phe-(Phe)-OH [TIP(P)], TIP(P)[ψ][17,18], H-Tyr-Tic-OH (Ref. 19), H-Tyr-Tic-Ala-OH and other Tyr-Tic tripeptides[19,20]. Although the measured δ affinities of the Tyr-Tic dipeptides were considerably weaker than TIP(P)[17], they represented the smallest opioid peptides to function as pure δ antagonists[19]. The development of these opioids has several advantages: a high hydrophobicity and low molecular weight, suitable for passage across the blood–brain barrier; limited conformation; and resistance to proteolytic degradation. Further modification of the dipeptides by replacement of Tyr with 2′,6′-dimethyl-L-tyrosine (Dmt) generated a series of remarkably potent and selective δ-opioid antagonists[21]. Building upon that prototypic design, a new generation of analogues has emerged[22–24] that provide paradigms for conformational studies[25–27] and serve as generalized lead compounds with future clinical and therapeutic applications in the development of new δ- and μ-opioid antagonists.

Opioid receptors

Before turning our attention to the main emphasis of this review, a brief summary of our current knowledge about opioid receptors and the purported δ-receptor binding domain will be useful in understanding the design of δ-selective ligands. The δ-, μ- and κ-opioid receptors belong to the extensive family of seven transmembrane G protein-coupled receptors and have many conserved features: consensus glycosylation sites in the N-terminal sequence; a disulfide bond between extracellular loops I and II; phosphorylation sites in the intracellular C-terminal region and on intracellular loops I and III; a palmitoylation site; and the involvement of the G proteins in linking the receptor with phospholipase C and G protein-coupled receptor kinases. Sequence similarity between disparate opioid receptors ranges from about 55 to 65%, with the main differences residing in the N- and C-terminal regions, and in the extra- and intracellular loops[28,29]. Site-directed mutagenesis, formation of chimeric receptor molecules[30–33] and molecular modeling studies[30,34] suggested that several residues in the transmembrane helices and extracellular loops, in particular the third extracellular loop[31–33], are part of the δ-receptor binding cleft. Although the extracellular N-terminal region does not appear to be involved in ligand binding, the C-terminal domain plays a role in sequestering and internalizing the receptor[35] and is also involved in opioid-induced desensitization, involving Thr and Glu residues and mediated by a dynamin-dependent endocytosis mechanism. Although pharmacological evidence revealed the existence of δ_1- and δ_2-receptor subtypes[29,36,37], only a single gene was found for each receptor family (δ, μ or κ)[28].

Opioid peptide agonists

Endogenous enkephalins, endorphins and dynorphins are agonists for δ-, μ- and κ-opioid receptors, respectively, and represent the initial framework upon which opioid antagonists were developed. Enkephalins exhibit relatively modest K_i values (5–10 nM) and low selectivities (<50 for δ receptors, relative to μ); by contrast, the endorphins and dynorphins, which represent C-terminal extensions of its tetrapeptide message core (H-Tyr-Gly-Gly-Phe), have specificity for μ and κ receptors, respectively[9]. Recently, the endomorphin tetrapeptides containing Pro in the second position (comparable with β-casomorphin, morphiceptin – an opioid peptide derived from the enzymatic degradation of β-casein – and Tyr-MIF-1) were shown to have high affinity and selectivity for the μ receptor[38].

Early structure–activity studies with enkephalins demonstrated that replacing Gly at the second position with D-Ala enhanced stability and activity[39]. Fortuitously, this anticipated the discovery of the amphibian skin opioid heptapeptides, which contain D-Ala or D-Met in that position[11]. However, the origin of the D-enantiomer, which is encoded by the codon for the L-isomer in the precursor cDNA (Ref. 40), remains unknown in vertebrates[41], although an enzyme catalyzing the formation of D-amino acids was recently characterized in yeast[42]. Amphibian opioids not only have the highest affinities and selectivities for δ or μ receptors of all naturally occurring opioid agonists, but also have resistance to proteolytic degradation[12–14]. Modification in the N-terminal

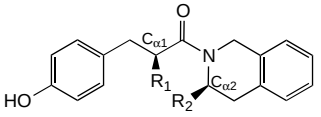

Figure 1. *Prototypic structure of δ-selective opioid antagonists.*

tri-[43–49] and tetrapeptide regions of deltorphin[44,48–51] led to new insights on physicochemical binding requirements and structural motifs of opioid peptide agonists. The N-terminal 'message domain' contains the sequences responsible for binding within the receptor site, whereas the C-terminal 'address domain' is considered to trigger the biological response[52].

The deduced structural requirements for a potent and selective δ-opioid agonist include the following:

- A phenolic side-chain (Tyr) as the N-terminal residue[9];
- An anionic residue (Glu or Asp) because neutral or cationic residues enhance interaction with μ receptors;
- A second aromatic center, Phe, which is common to all opioid peptides[9,11];
- A protonated nitrogen at the N-terminus;
- A D-isomer in the second position to restrict peptide conformation[53] and biological stability[13];
- A hydrophobic region that is associated with residues in the C-terminal address domain[54].

The design and formation of small δ-opioid antagonists initially began with the manipulation of the μ-selective dermorphin tetrapeptide agonists (Tyr-D-Xaa-Phe-Yaa)[43,55] in which the D-isomer in the second position and the residue in the fourth position were replaced by aromatic residues in order to build a fully aromatic ligand while retaining the protonated nitrogen and negative charge at the N- and C-termini, respectively[17,18]. From the discovery of these δ antagonists, consecutive aromatic pharmacophores were considered responsible for receptor interaction, as seen in the development of the δ-opiate antagonist naltrindole from naloxone[56]. Nonetheless, subsequent studies on these Tic[2]-containing antagonists provided the basis for the strategy employed in formulating δ-opioid dipeptide antagonists.

Design strategies for δ-opioid antagonists

The design and synthesis of δ-opioid antagonists maintained properties that are known to be important for high affinity and receptor selectivity in δ agonists, and included a consideration of the chirality at $C_{\alpha 1}$ and $C_{\alpha 2}$. Furthermore, the deletion of Phe residues in TIPP provided new awareness on the minimal requirement for an active δ antagonist[19].

First generation of δ antagonists

A major step in the development of δ-selective opioid antagonists was the incorporation of Tic in tri- (TIP and TIP[ψ]) and tetrapeptides (TIPP and TIPP[ψ])[17,18] (Figure 1). The importance of Tic is threefold: to constrain the peptide backbone dihedral torsion angle ϕ (similar to proline), to restrain the side-chain, and to provide an additional aromatic and hydrophobic residue in the peptide. Substitution of L-Tic[2] in [Leu[5]]enkephalin, dermorphin[57] and the κ-ligand dynorphin A (1–11)[58] converted these peptide agonists into antagonists, and remarkably, the μ agonist dermorphin became a δ antagonist[57].

To prevent peptide cyclization that occurs under acidic conditions[59–61], a reduced (CH_2-NH) bond was introduced into TIP[ψ] and TIPP[ψ] (Ref. 18), allowing Tic to assume a distinct conformation, relative to the phenol ring of tyrosine[62,63].

The aromaticity afforded by Phe was considered essential for opioid tetra- to heptapeptides[9,46,47,64]; its elimination from TIP(P) led to the development of di- (**1**,**2**)[19] and tripeptide antagonists (**5**,**6**; Table 1)[19,20]. The importance of Tic, however, was further investigated through systematic replacement by aromatic, heterocyclic, Pro or 1-aminocyclohexane residues in numerous di- and tripeptide analogues; all changes were ineffective in producing high-affinity δ antagonists (data not shown; see below), suggesting that the aromatic ring of Tic was necessary for δ-opioid receptor recognition.

Structural modification of the Tyr-Tic pharmacophore

Change in chirality at $C_{\alpha 1}$ of Tyr and $C_{\alpha 2}$ of Tic in di- and tripeptides was detrimental for δ-receptor recognition; similar changes in chirality of each amino acid in deltorphin led to adverse effects[44]. Amidation of the dipeptide exerted minimal effect on δ affinity; however, it enhanced binding to μ receptors, particularly in combination with D-Tic (**3**) in TIP(P)[17], resulting in the unusual reversal of receptor selectivity[17,18,21]. Because studies on opioid agonists established that a free-acid function is strictly required for δ selectivity but not δ affinity, it was surprising

Table 1. Structure–activity profiles of δ-selective opioid antagonists containing Tyr-Tic

No.	Substituents		Chirality		Binding K_i (nM)		
	R_1	R_2	$C_{\alpha1}$	$C_{\alpha2}$	δ	μ	μ/δ
1	$-NH_2$	-COOH	L	L	192	28,400	148
2	$-NH_2$	$-CONH_2$	L	L	166	28,700	173
3	$-NH_2$	$-CONH_2$	L	D	6,000	3,100	0.5
4	$-NH_2$	$-CH_2OH$	L	L	128	11,000	86
5	$-NH_2$	-CO-Ala-OH	L	L	56	8,300	148
6	$-NH_2$	-CO-Ala-NH_2	L	L	55.6	33,800	608
7	$-NH_2$	-CO-D-Ala-NH_2	L	L	67.1	26,940	401
8	$-NH_2$	-CO-Ala-Ala-OH	L	L	54.5	117,000	22
9	$-NH_2$	-CO-Asp-NH_2	L	L	69.8	46,500	664
10	$-NH_2$	-CO-Arg-NH_2	L	L	281	5,390	19
11	$-NH_2$	-CO-Ala-Arg-NH_2	L	L	22.1	1,835	83
12	$-NH_2$	-CO-Ala-Ala-Arg-NH_2	L	L	8.8	4,291	488
13	$-NH_2$	-CO-Ala-OCH_3	L	L	24	5,120	211
14	$-NH_2$	-CO-Ala-NH-adamantane	L	L	3.1	1,500	488
15	$-NH_2$	-CO-NH-adamantane	L	L	54.4	105	2
16	$-NH_2$	-CO-Ala-$NHCH_3$	L	L	3.3	4,015	1,217
17	$-NHCH_3$	-COOH	L	L	74.7	12,386	166
18	$-N(CH_3)_2$	-COOH	L	L	43.5	11,523	265

to find that reducing the carboxylate to an alcohol (**4**) brought inconsequential changes in δ and μ affinities. The formation of a tripeptide by incorporation of Ala (**5**–**7**,**13**,**14**,**16**) or Asp (**9**) in the third position consistently elevated δ affinity, relative to the Tyr-Tic dipeptides (**1**,**2**). These enhanced properties were increased in tetrapeptides containing Ala (**8**) or Arg (**11**), or a pentapeptide with Ala-Arg (**12**). Phenylalanine[17] or leucine[20] in the third position also elevated receptor binding.

Efforts to enhance the hydrophobicity of the Tyr-Tic peptides produced two additional groups of analogues. The first consisted of C-terminally modified tripeptides containing a methyl ester (**13**), an adamantanyl amide (**14**) or a methyl amide (**16**), in which both δ binding and δ selectivity increased (**14**,**16**), while a dipeptide adamantanyl amide (**15**) dramatically increased μ affinity. The second group, the *N*-methylated analogues, forming secondary (**17**) or tertiary amines (**18**), had marginally different δ-receptor binding properties in comparison to the parent peptide (**1**). Unfortunately, none of the manipulations of Tyr-Tic pharmacophore analogues produced peptides with high affinity (<1 nM) or high selectivity (>1,000).

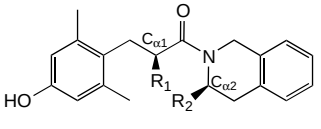

Figure 2. *Prototypic structure of Dmt-Tic-containing opioid antagonists. Dmt, 2′,6′-dimethyltyrosine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.*

A hypothesis proposed that increased hydrophobicity and restriction of the conformational freedom of Tyr might enhance the biological effectiveness of opioid peptides[65]. To test that idea, hydrophobic derivatives of Tyr were initially incorporated into a variety of analogues that yielded bioactive molecules, lacking receptor selectivity[66–70]. By contrast, modifications of Tyr that increased the hydrophobicity of Tyr-Tic peptides resulted in derivatives with high δ-receptor selectivity.

Design of new lead compounds: augmented hydrophobicity

Substitution of Tyr by Dmt

Incorporation of Dmt (synthesized and generously supplied by J.H. Dygos[71]) yielded H-Dmt-Tic-OH (**19**) (Figure 2), which elevated δ affinity by nearly five orders of magnitude and increased selectivity 1,000-fold (Table 2). This new lead compound had full δ-antagonist bioactivity *in vitro*[21] (Table 5) and *in vivo* following either i.c.v. administration[72] or systemic (i.p., s.c. or i.v.) injection[73]. Relevance of the Dmt-Tic pharmacophore is evident in the heightened activity of the Dmt-containing peptides. The

Table 2. Structure–activity profiles of δ-selective opioid antagonists containing Dmt-Tic

No.	Substituents		Chirality		Binding K_i (nM)		
	R_1	R_2	$C_{\alpha1}$	$C_{\alpha2}$	δ	μ	μ/δ
19	-NH_2	-COOH	L	L	0.02	3,320	150,800
20	-NH_2	-$CONH_2$	L	L	1.2	277	227
21	-NH_2	-COOH	L	D	14	224	17
22	-NH_2	-$CONH_2$	L	D	57	4.0	0.07
23	-NH_2	-CH_2OH	L	L	0.4	151	344
24	-NH_2	-CO-Ala-OH	L	L	0.29	5,800	20,400
25	-NH_2	-CO-Ala-NH_2	L	L	0.24	47	195
26	-NH_2	-CO-Ala-OH	L	D	88.7	82.8	0.9
27	-NH_2	-CO-Ala-NH_2	L	D	46.4	5.8	0.13
28	-CH_3	-COOH	L/D	L	1,300	71,400	55
29	-NH_2	-COOH	D	L	124	10,960	88
30	-NH_2	-CO-Ala-OH	D	L	13	2,560	197
31	-$NHCH_3$	-COOH	D	L	3.2	2,000	628
32	-$NHCH_3$	-COOH	L	L	0.20	380	1,900
33	-$NHCH_3$	-$CONH_2$	D	L	11	1,330	119
34	-$NHCH_3$	-$CONH_2$	L	L	0.38	955	2,500
35	-$NHCH_3$	-CO-Ala-OH	L	L	0.14	5,850	41,800
36	-$NHCH_3$	-CO-Ala-OH	D	L	106	19,210	181
37	-$N(CH_3)_2$	-COOH	L	L	0.12	2,440	20,300
38	-$N(CH_3)_2$	-$CONH_2$	L	L	0.31	511	1,650
39	-$N(CH_3)_2$	-COOH	D	L	5.7	13,100	2,280
40	-$N(CH_3)_2$	-CO-Ala-OH	L	L	0.076	1,520	20,000
41	-$N(CH_3)_2$	-CO-Ala-OH	D	L	108	18,960	176
42	-$NHCOCH_3$	-COOH	L	L	270	45,490	169
43	-$NHCOCH_3$	-$CONH_2$	L	L	115	14,900	130
44	-$NHCOCH_3$	-CO-Ala-OH	L	L	308	5,060	16
45	-$NHCOCH_3$	-CO-Ala-NH_2	L	L	62	35,600	573
46	-NH_2	-CH_2COOH	L	L	0.84	418	498
47	-NH_2	-$CONHNH_2$	L	L	0.99	85.1	86
48	-NH_2	-$CONHCH_3$	L	L	0.47	85.5	182
49	-NH_2	-$COOCH_3$	L	L	9.6	423	44
50	-NH_2	-CONH-adamantane	L	L	0.26	0.76	3.0
51	-NH_2	-CONH-adamantane	L	D	24.5	0.26	0.01
52	-NH_2	-CO-Ala-NH-adamantane	L	L	0.073	2.52	35
53	-$N(CH_3)_2$	-CONH-adamantane	L	L	0.16	1.12	7.0
54	-$N(CH_3)_2$	-CONH-adamantane	L	D	139.7	50.5	0.36
55	-*N*-piperidine	-COOH	D	L	59.1	6,540	111
56	-*N*-piperidine	-COOH	L	L	1.18	2,039	1,728
57	-*N*-pyrrolidine	-COOH	D	L	53.4	2,020	38
58	-*N*-pyrrolidine	-COOH	L	L	1.62	814	502
59	-*N*-pyrrole	-COOH	D/L	L	336	9,184	27
60	-*N*-pyrrole	-COOH	L	L	15.0	5,882	392
61	-CN	-COOH	D/L	L	4,300	8,360	2.0
62	-$N(CH_2CH_3)_2$	-COOH	D	L	11.6	6,052	522
63	-$N(CH_2CH_3)_2$	-COOH	L	L	0.92	35.3	38
64	-NH_2[a]	-COOH	L	L	6.7	3,200	486
65	-NH_2[b]	-COOH	L	L	7.7	1,640	213
66	-NH_2	-COOH	D/L	L	0.46	1,158	2,517
67	-CH_2NH_2	-COOH	L	L	301	4,489	15
68	-NH_2	-CONH-tetrazole	L	L	0.70	37	53

[a] 3'5'-diiodo-Dmt

[b] Monoiodo-Dmt (3' and 5')

increased hydrophobicity and alteration in conformation might enhance receptor interaction through π–π stacking, stabilization of hydrophobic interactions with aliphatic or aromatic side-chains in the receptor, and strengthen hydrogen bonding capabilities of the hydroxyl group, as suggested from site-directed mutagenesis studies of the δ receptor[30,31,33] and molecular modeling[34].

Enhancement of hydrophobicity

Further enhancement of the hydrophobic properties of H-Dmt-Tic-OH (**19**) produced ligands with variable δ-opioid antagonist activities. For example, the amidation of the di- (**20**,**22**,**33**,**34**,**38**) and tripeptides (**25**,**27**), or reduction of the acid to an alcohol (**23**), enhanced μ binding with only modest changes in K_iδ. The largest variation in affinities and selectivities upon amidation occurred in conjunction with D-Tic[2], in which the di- (**22**) or tripeptide (**27**) became μ selective. This observation provided evidence on their potential application as bifunctional probes for δ and μ receptors[74].

In both non-alkylated peptides (**24**–**37**,**30**) and *N*-alkylated peptides (**35**,**36**,**40**,**41**), alanine in the third position further elevated hydrophobicity while maintaining high δ affinity and selectivity when $C_{\alpha 1}$ and $C_{\alpha 2}$ were in the L-configuration (compare the peptide pairs **24** with **36**, **25** with **27**, **35** with **36**, and **40** with **41**). All compounds were potent antagonists *in vitro* (Table 5)[21]. Whereas i.c.v. administration of the tripeptide (**24**) yielded *in vivo* antagonist activity, it failed to demonstrate antagonism when administered systemically[73]. Comparable with dipeptides, the receptor binding of tripeptides was also sensitive to amidation, which produced a dramatic loss in δ selectivity through a conspicuous gain in μ affinity (**25**), especially in the μ-selective D-Tic[2] analogue (**27**).

Hydrophobic modifications at the N- and C-termini

Increasing hydrophobicity by the addition of uncharged and lipophilic N- and C-terminal substituents provided an awareness of the spatial requirements of a δ antagonist within the receptor pocket and on the nature of δ- and μ-opioid receptors. At the N-terminus, *N*-mono- and *N,N*-dialkylation by methyl groups in the di- (**31**–**34**,**37**–**39**) and tripeptides (**35**,**36**,**40**,**41**) rendered several compounds with high δ affinity and selectivity (Table 2). By contrast, *N*-alkylation by diethyl (**38**,**39**), piperidine (**55**,**56**), pyrrolidine (**57**,**58**) or pyrrole groups (**59**,**60**) generally diminished the high binding capacity of the ligand, reducing its selectivity.

The C-terminal hydrazide (**47**), methyl amide (**48**) and methyl ester (**49**) derivatives of Dmt-Tic decreased δ affinity while elevating μ affinity (Table 2). The tetrazole (**68**), as an isostere of the carboxylic function, did not sufficiently reduce interaction with μ-opioid receptors, thereby reducing δ selectivity. This behaviour differs from angiotensin antagonists, where a tetrazole can replace a carboxylic group without affecting activity[75]. The inclusion of a bulky 1-adamantanyl amide group (with its high hydrophobic, steric constants and van der Waals volume[76]) into dipeptides (**50**,**51**,**53**,**54**) and a tripeptide (**52**), intensified μ-opioid receptor interaction without significant alteration of δ affinity, except for compound **54**, which lost δ affinity and selectivity.

Further enhancement of hydrophobicity by mono- (**65**) or diiodination (**64**), ortho to the hydroxyl group of Dmt, negatively affected δ-binding properties. The bulky iodine atoms might sterically hinder the hydrogen bonding potential of the hydroxyl group between the ligand and receptor. Relative to the prototypic dipeptide (**19**), the addition of another methyl group by the incorporation of β-methyl-2′,6′-dimethyl-L-tyrosine (Tmt), however, resulted in a 400-fold loss of δ-receptor binding and drop in selectivity by 40-fold[77].

Spatial modifications of the charged centres in the Dmt-Tic pharmacophore

Incorporating a methylene group between $C_{\alpha 1}$ and the protonated amine of Dmt at the N-terminus (**67**), or between $C_{\alpha 2}$ and the free-acid function of Tic at the C-terminus (**46**), altered the flexibility of the acid function required for distinguishing between δ and μ receptors. In any case, compound **46** maintained moderate affinity with δ receptors while avoiding diketopiperazine formation. However, the loss in selectivity was greater than with the addition of Ala (**24**) (Table 2), verifying the requirement of an aliphatic side-chain at the third position[20] and providing further evidence that the dipeptide represents an optimum size for a δ-opioid antagonist. The observations that a protonated nitrogen is necessary in close proximity to the phenolic side-chain of tyrosine[78] or Dmt was seen in the reduced affinity of analogues with a methylene bridge at the N-terminus (**67**), and those in which the amino function was replaced by a nitrile group (**61**) or was acetylated (**42**–**45**).

Pharmacophores lacking Tic

To determine whether Tic was solely responsible for

δ-receptor antagonism, substitution analogues in the second position (Figure 3) were investigated by varying aromaticity, side-chain composition, chirality and other conformational constraints. In a large number of analogues tested, replacement by N^{α}-methyl-Phe (as an open ring analogue of Tic), Spi (4,5,6,7-tetrahydro-1H-imidazole[4,5-c]pyridine-6-carboxylic acid), Azp (4-amino-1,2,4,5-tetrahydrobenzo[c]azepin-3-one), Ac_6c (1- aminocyclohexane-1-carboxylic acid), Aic (2-aminoindan-2-carboxylic acid), ψ(CN_4) (1,5-disubstituted-1H-tetrazole), Trp or Pro were completely inactive (data not shown).

Of the remaining peptides, only a few Dmt-Phe compounds weakly interacted with opioid receptors (**69–76**) and these are shown in Table 3; however, they behaved not as δ antagonists, but rather surprisingly as μ antagonists with relatively good activity *in vitro*, such as that seen with H-Dmt-D-Phe- NH_2 (**72**) (Table 5)[79]. The D-Phe analogue (**72**) augmented μ binding[79] relative to the title compound (**19**) by an astounding 650,000-fold increase in μ selectivity. Therefore, as seen with the Dmt-Tic analogues, the chirality at $C_{\alpha 1}$ with Dmt (**70**) or $C_{\alpha 2}$ for Phe (**72**,**76**) or both residues (**71**,**75**) directed affinity toward one receptor type or another which defined selectivity. Interestingly, N^{α}-methylation of Phe inactivated the peptide (**74**) in comparison to H-Dmt-Phe-O-CH_3 (**73**). The [Dmt^1,Phe^2]penta-

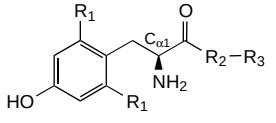

Figure 3. *Diagrammatic structure of opioid antagonists lacking Tic[2]. Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.*

peptide analogues lacking an acidic group exhibited relatively high μ affinity and selectivity[79], analogous to the weak μ selectivity of morphiceptin and C-terminal deletion analogues of deltorphin.

Diketopiperazine analogues

The formation of diketopiperazine can occur spontaneously under the acidic conditions during peptide synthesis and storage in peptides containing imino acids such as Pro or Tic (Refs 59,61,80). Several natural diketopiperazines are biologically active, including the morphiceptin-derived *cyclo*(Tyr-Pro), which had modest μ affinity and weak agonist bioactivity[81], comparable to *cyclo*(Dmt-Tic) (**77**) (Figure 4) for δ receptors (Tables 4 and 5). Of course, *cyclo*(Dmt-Tic) with a $K_i\delta$ = 10 nM and δ selectivity of 66 cannot be considered an exceptionally good opioid when compared to the title dipeptide (**19**) or many of its active derivatives (Table 2). However, the key implication is that this peptide, which lacks a protonated nitrogen, interacts with δ receptors while the *cyclo*(Tyr-Tic) cognates (**80**,**81**) are completely *inactive*. These data further substantiate the importance of the methyl groups on the phenol ring of Dmt to align the other ligand substituents with the receptor-binding domain, suggesting that the methyl groups influence the solution conformation of a peptide[25,26]. On the other hand, the weak *in vitro* antagonism of *cyclo*(Dmt-Tic) implies that a protonated nitrogen is probably involved in anchoring the peptide firmly in receptors in peripheral tissue[24]. Only two other diketopiperazines (**82**,**83**) of a dozen analyzed demonstrated nonselective interaction with δ and μ receptors and $K_i\delta$ values in the range of 50–150 nM. Because the analogues H-Tic(7-OH)-Tic-Ala-OH and its dipeptide diketopiperazine were inactive, the data support the requirement for proper spatial orientation of the hydroxyl group to interact with the

Table 3. Structure–activity profiles of opioid peptides lacking Tic

No.	Substituents			Chirality		Binding K_i (nM)		
	R_1	R_2	R_3	$C_{\alpha 1}$	$C_{\alpha 2}$	δ	μ	μ/δ
69	-CH_3	-Phe	-NH_2	L	L	387	210	0.5
70	-CH_3	-Phe	-NH_2	D	L	1,977	866	0.4
71	-CH_3	-Phe	-NH_2	D	D	2,543	501	0.2
72	-CH_3	-Phe	-NH_2	L	D	15.5	3.6	0.23
73	-CH_3	-Phe	-OCH_3	L	L	122	46	0.38
74	-CH_3	-$N^{\alpha}(CH_3)$-Phe	-OCH_3	L	L	1,343	2,870	1.7
75	-CH_3	-Phe	-OH	D	D	3,516	5,308	1.5
76	-CH_3	-Phe	-OH	L	D	24.2	659	27

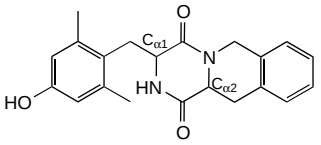

Figure 4. *Structure of cyclo(Dmt-Tic) opioid diketopiperazine. Dmt, 2',6'-dimethyltyrosine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.*

receptor. Nonetheless, diketopiperazines offer a rigid scaffold for the generation of new classes of opioid antagonists that lack charged groups and possess inherently higher hydrophobic properties.

Conformational studies

Antagonist pharmacophores

Several related models were proposed for the interaction of antagonists with δ receptors based on proton nuclear magnetic resonance spectroscopy (^{1}H-NMR)[19,26,82], X-ray crystallography[62,83] and molecular modeling approaches using H-Tyr-Tic-NH_2, H-Dmt-Tic-NH_2 (Refs 19,25,84–86) and H-Dmt-Tic-OH (Ref. 27). A prevalence of a *cis* over a *trans* peptide bond between Tyr[1] and Tic[2] in H-Tyr-Tic-NH_2 was observed[19] and supported by others[85,86]; the *gauche* (+) orientation was also observed with a low-energy conformation of Tmt-Tic-OH (Ref. 77). Studies on TIP(P) and TIP(P)[ψ] suggested that a presumed 'receptor-bound conformation' involved a 'sandwich' (parallel) arrangement of the Tic[2] and Phe[3] aromatic rings[85]; however, whether Tyr[1] is tilted or coplanar, relative to naltrindole, remains unanswered[85]. An analogous model was postulated for the parallel placement of the aromatic rings of Dmt and Tic in a low-energy model of *cyclo*(Dmt-Tic)[25] as well as H-Dmt-Tic-OH (Ref. 27; Figure 5). In another proposed bioactive conformation of *cyclo*(Dmt-Tic), the aromatic rings of Dmt and Tyr were orthogonal[26]. The parallel conformers in solution determined by ^{1}H-NMR were comparable to a single conformation of the inactive *cyclo*(Tyr-Tic) (**80**) obtained by X-ray crystallography[82].

Conformationally restricted dipeptide antagonists

Different methodological approaches deduced similar models for *cyclo*(Tyr-Tic) (**80**)[82] and *cyclo*(Dmt-Tic) (**77**)[25,26]. A theoretical model of *cyclo*(Dmt-Tic)[25] was based on superimposition of low energy structures on the

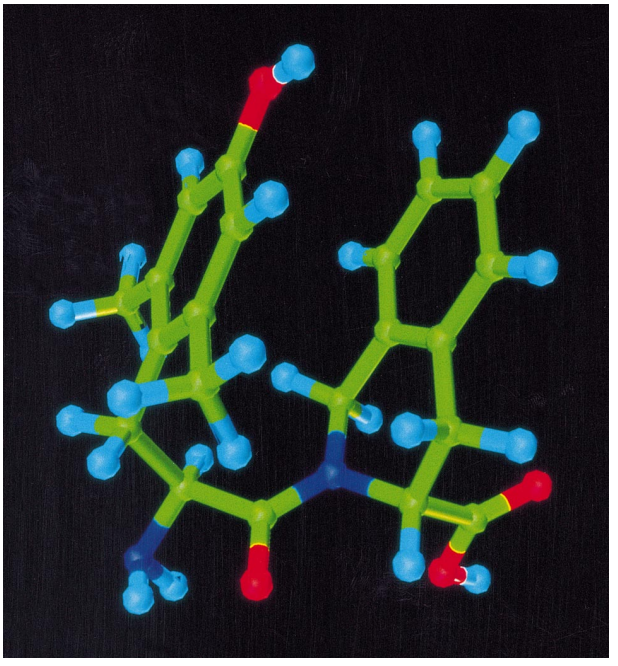

Figure 5. *Low-energy model of Dmt-Tic-OH representing the pharmacophore features of a prototypic δ-opioid receptor antagonist. Those features are close proximity and approximate parallel orientation of the aromatic rings,* cis *orientation of the peptide bond (N-C′) and* guache$^-$ *(−60°) and* guache$^+$ *(60°) orientation about C_α-C_β bonds of Dmt and Tic, respectively. Carbon, hydrogen, oxygen and nitrogen atoms are represented by green, light blue, red, and dark blue, respectively. Dmt, 2′,6′-dimethyltyrosine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.*

crystalline structures of *cyclo*(Tyr-Tic)[82], H-Tyr-Tic-NH_2 (**2**) (Flippen-Anderson, J.L., pers. commun.) and naltrindole, while another study[26] superimposed *cyclo*(Dmt-Tic) only on *N*-methylnaltrindole. The major distinction among these three-dimensional models was the distance between the aromatic rings of Dmt and Tic.

The aromatic ring-to-ring distance within the peptide could, in fact, be important in the 'receptor-bound conformation' of δ-opioid antagonists and provides a basis for the development of additional lead compounds. It might also explain the differences in activity between H-Dmt-Phe-OH (or H-Tyr-Phe-OH) and H-Dmt-Tic-OH and TIP(P) peptides, because Phe would have greater degrees of rotational freedom compared to Tic. The 5.4 Å aromatic ring-to-ring distance in one structure derived from Monte Carlo

Table 4. Activity profiles of diketopiperazines

No.	Compound	Binding K_i (nM) δ	μ	μ/δ
77	c(Dmt-Tic)	9.58	635	66
78	c(Dmt-D-Tic)	499	728	1.5
79	c(D-Dmt-Tic)	284	48,876	172
80	c(Tyr-Tic)	41,848	51,474	1.2
81	c(Tyr-D-Tic)	8,908	127,602	14
82	c(Dmt-Phe)	176	135	0.8
83	c[N^{α}(CH_3)Dmt-Tic]	57.4	61.2	1.1

conformational searching for low-energy cluster I of *cyclo*(Dmt-Tic)[25] was remarkably similar to that of crystalline TIP(P), in which the distance between Tic^2 and Phe^3 residues was 4.2 Å; an even greater similarity is seen in the 5.9 Å distance between Tyr^1 and Tic^2, owing to the folded nature of the peptide backbone[63]. These aromatic ring-to-ring distances were comparable to the 4.9 Å distance found in a crystalline analogue of the enkephalin antagonist ICI174864 (*N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH)[63], but somewhat shorter than the theoretically obtained 6.3–6.5 Å (Ref. 85) and 6–8 Å for TIP(P) (Ref. 86). An interesting conformational observation is that the distance found in opioid antagonists is less than half of that in agonists[62,87]. Thus, not only is the *cis* or *trans* orientation around the second residue important, but the distance between aromatic substituents might be considered another distinctive feature that differentiates δ antagonists from δ agonists[51,88] and distinguishes them from μ agonists, which have even more extended topographies[89] and different orientation.

Compact shapes were reported for H-Dmt-Tic-NH_2 (**19**), and the lowest energy models with *cis* peptide bonds for this peptide superimposed more satisfactorily on *N*-methylnaltrindole than those with *trans* orientations. Similarly, *cis* conformers of H-Tyr-Tic-NH_2 (**2**)[90], TIP(P) and TIP(P)[ψ] superimposed well with naltrindole[85]; however, the aromatic rings of Tyr or Dmt determined by ^{1}H-NMR failed to superimpose with corresponding atoms

Table 5. Functional biological activities of Tyr-Tic, Dmt-Tic and Dmt-Phe peptides[a]

No.	Sequence	MVD (nM) pA_2	K_e	IC_{50}	GPI (μM) pA_2	K_e	IC_{50}	GPI/MVD
2	H-Tyr-Tic-NH_2	6.0	1,000	–	–	–	>10	>10
3	H-Tyr-D-Tic-NH_2	–	–	>10,000	–	–	>10	–
5	H-Tyr-Tic-Ala-OH	7.0	100	–	–	–	>10	>100
6	H-Tyr-Tic-Ala-NH_2	6.2	631	–	–	–	>10	>16
19	H-Dmt-Tic-OH	8.2	6.3	–	–	–	>10	>1,600
20	H-Dmt-Tic-NH_2	7.2	63	–	–	–	>10	>160
23	H-Dmt-Tic-CH_2OH	7.0	100	–	–	–	>10	>100
24	H-Dmt-Tic-Ala-OH	8.4	4.0	–	–	–	>10	>2,500
25	H-Dmt-Tic-Ala-NH_2	8.0	10	–	–	–	>10	>1,000
30	H-D-Dmt-Tic-Ala-OH	6.9	125	–	–	–	>10	>80
31	H-*N*(CH_3)-D-Dmt-Tic-OH	6.9	125	–	–	–	>10	>80
32	H-*N*(CH_3)-Dmt-Tic-OH	8.5	3.2	–	–	–	>10	>3,100
34	H-*N*(CH_3)-Dmt-Tic-NH_2	8.6	2.5	–	–	–	>10	>4,000
35	H-*N*(CH_3)-Dmt-Tic-Ala-OH	8.8	1.6	–	–	–	>10	>6,250
37	*N,N*(CH_3)$_2$-Dmt-Tic-OH	9.4	0.28	–	5.8	1.58	–	5,640
38	*N,N*(CH_3)$_2$-Dmt-Tic-NH_2	9.9	0.12	–	6.3	0.54	–	4,500
39	*N,N*(CH_3)$_2$-D-Dmt-Tic-OH	8.6	2.5	–	–	–	>10	>4,000
40	*N,N*(CH_3)$_2$-Dmt-Ala-Tic-OH	9.6	0.22	–	5.8	1.58	–	7,180
43	H-*N*(CH_3CO)-Dmt-Tic-NH_2	6.5	316	–	–	–	>10	>30
69	H-Dmt-Phe-NH_2	IA	IA	IA	IA	IA	IA	–
70	H-D-Dmt-Phe-NH_2	IA	IA	IA	IA	IA	IA	–
71	H-D-Dmt-D-Phe-NH_2	IA	IA	IA	4.9	1.2×10^{-4}	–	–
72	H-Dmt-D-Phe-NH_2	IA	IA	IA	6.5–7.2[b]	63–316	–	–
73	H-Dmt-Phe-Gly-Val-Val-NH_2	IA	IA	IA	5.7	–	–	–
77	*cyclo*(Dmt-Tic)	5.4	3,980	–	–	–	20	5

[a] IA, Inactive (>10^{-4} M).

[b] Antagonism against μ receptors varied according to the μ agonist used [(D-Ala^2, *N*-methyl-Phe^4,Gly-ol^5)-enkephalin (DAGO), morphine or dermorphin].

in *N*-methylnaltrindole[90].

Substituents involved in receptor interaction

Hypothetical models of these δ antagonists were postulated in terms of their conformation and proposed attachment points within the receptor. One model[86], based on TIP-NH_2, hypothesized the involvement of the nitrogen of the Tyr^1 amine and the hydrophobic centres of Tyr^1 and Tic^2 or Phe^3. This model is reminiscent of the T-shaped molecule developed for dermorphin tetrapeptides (based on a comparison to the rigid alkaloid fentanyl) that consists of a hard and soft base, and a proper orientation of the two pharmacophoric aromatic residues[78]. The other concept developed several points of attachment between the peptide and receptor: data on *cyclo*(Dmt-Tic) (**77**)[24] suggested that hydrophobic interactions are embodied in the aromatic rings of Dmt and Tic and methyl groups of Dmt, while hydrogen bonding involves interaction by the hydroxyl group on Dmt, and the aromatic residues are involved in cation-π interactions[91]. Methyl groups at the 2′ and 6′ positions of the phenol ring might also restrict rotational freedom in the receptor cleft to permit greater stabilization of the peptide.

Conclusion

Recent years have seen rapid progress in the development of small, highly potent and δ-selective opioid peptide antagonists that are resistant to biodegradation, pass through the blood–brain barrier *in vivo*[73] and act as pharmacological probes of opioid receptors[21,23]. These δ antagonists evolved progressively from studies on the N-terminal analogues of amphibian peptides[43,92,93], resulting in TIP(P) and TIP(P)[ψ][17,18], the Tyr-Tic di- (**1**,**2**)[19] and tripeptides (**5**,**6**) lacking phenylalanine[19,20]. The dipeptides remain the smallest compounds that bind to δ-opioid receptors and produce antagonism[19,21], which is now confirmed by others[77]. While another dipeptide exists, H-Tyr-Arg-OH (kyotorphin), it is known to interact poorly with μ-opioid receptors and primarily reinforces the activity of endogenous opioids[94].

The successful strategy that enhanced affinity and biological potency by several orders of magnitude was substitution of Dmt for Tyr (Ref. 21). From this prototypic molecule there emerged an interesting array of new compounds (Table 2), including *N*-alkylated derivatives, which increased biological activity by 10- to 20-fold (Table 5), C-terminally modified analogues (Table 2), and a highly hydrophobic diketopiperazine (Table 4). Furthermore, the small size, overall shape, electrostatic charge distribution and hydrophobic nature associated with the Dmt-Tic pharmacophore compounds are advantageous features for therapeutic applicability. Their potential clinical application involves the ability to elicit δ antagonism after systemic administration *in vivo*[73] because small δ-opioid antagonists are known to act centrally[72,95].

The C-terminal adamantanyl amide derivative, with its elevated hydrophobic constant, localized electric effect and steric constant[76], could have permeability comparable to that of the narcotic opiates, and possibly a dual role at both δ- and μ-opioid receptors. Compounds of immediate interest for clinical trials include *N*-alkylated- (**32**,**34**,**35**,**37**–**40**) and C-terminally modified Dmt-Tic (**50**–**54**), which may augment the current regime of drug(s) available in the armoury against syndromes in which δ-opioid receptors are implicated: for example, alcohol dependency[96,97] and cocaine[2,98] and morphine addiction[99]. Another important application involving δ-opioid receptors includes immunosuppression[100] during organ transplantation[5] through the modulation of interleukin levels[101]. Thus, dipeptide δ antagonists could be administered through intravenous injection, nasal inhalation or oral administration with or without encapsulation in biodegradable polymers[102]. Despite the selectivity of these antagonists, the attenuation of tolerance and dependence to morphine through antagonism of a δ receptor[95] cannot be underestimated and could be clinically important for their future therapeutic efficacy.

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