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Letter

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Chiral Effect of a Phe Residue in Position 3 of the Dmt[1]-L(or D)-Tic[2] Analogues on Opioid Functional Activities

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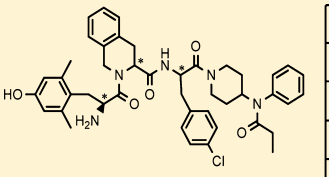
Supporting Information

ABSTRACT: In this letter, we describe a structure−activity relationships study, specifically related to the chirality of third amino acid residue in our H-Dmt-L(or D)-Tic analogues, of which C-terminus is attached to a piperidinyl moiety. Observed selectivities and functional activities of these analogues demonstrated that the chiralities of the second and third position residues are crucial for determining whether these ligands act as antagonists or agonists at the δ opioid receptor, but not at the μ opioid receptor.



L-Dmt-L(or D)-Tic-L(or D)-Phe(*p*-Cl)-piperidinyl

chirality	MVD	functional activity
L, L, L	K_e=0.79±0.6 nM	δ antagonist
L, L, D	IC_{50}=74±14 nM	δ agonist
L, D, L	K_e=150±20 nM	δ antagonist
L, D, D	0% at 1 μM	no function

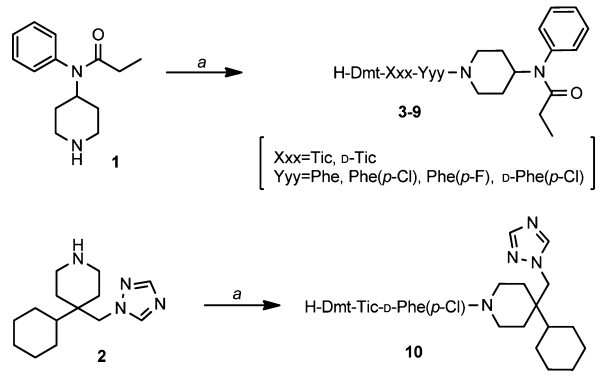
KEYWORDS: *Dmt-Tic, opioid functional activities, structure−activity relationship, δ opioid receptor, chirality*

Dmt-Tic has been known as the most potent δ opioid receptor antagonist with high selectivity and a minimum peptide unit.[1] In many studies, modifications of Dmt-Tic at the C-terminus changed the biological profile dramatically, for example, from selective δ opioid receptor antagonist to mixed μ/δ opioid receptor agonists.[2−5] These big changes in the biological profile might be the result of topographical changes caused mainly by the conformational preference of the Tic residue resulting from the modifications because it has been known that close proximity and parallel orientation of the two aromatic rings of Dmt-Tic are key features of δ opioid antagonist activity.[2,6,7]

In our recent studies, we observed that the attachment of a D-Phe(*p*-Cl) to the dipeptide structure in analogue **5** changed opioid receptor function from a δ antagonist to a δ agonist.[8] This result suggests a profound influence of D-amino acid substitution in position 3 on the potency of Dmt-Tic analogues. It is certainly possible that the bulky aromatic group of the D-Phe(*p*-Cl) residue can cause a conformational change of the Tic residue, which is responsible for the δ opioid receptor antagonist activity. It is also possible that the chirality of the third amino acid residue plays a role in a conformational change of the Tic residue because analogues in which two Phe residues are attached to the C-terminus of Dmt-Tic (H-Dmt-Tic-Phe-Phe-OH and H-Dmt-Tic-Phe-Phe-NH_2) still possess antagonist activities at the δ opioid receptor (K_e = 0.20 and 0.21 nM, respectively).[9,10]

On the basis of these observations of the chiral effect of the Phe residue, we have further investigated the chiral effect of the Phe[3] residue with a series of analogues in which the configurations of Tic and Phe(*p*-Cl) (or Phe, or Phe(*p*-F)] residues are D or L and the C-terminus is linked to the piperidinyl moieties **1** or **2** (Scheme 1 and Table 1).

Scheme 1. Synthesis of Dmt-L(or D)-Tic Analogues[a]



[a]Reagents and conditions: (a) stepwise coupling (1.1 equiv BOP/1.1 equiv HOBt/2.5 equiv NMM, DMF, room temperature, 2−3 h), followed by deprotection (TFA, 0 °C, 20 min).

Thanks to the piperidinyl moieties and the Dmt residue, this series of analogues exhibits high lipophilicity (aLOGPs of 4.02−4.91 in Table 1), which is a key factor for penetrating the blood−brain barrier (BBB). All analogues were evaluated for their affinity and selectivity for μ and δ opioid receptors using transfected HN9.10 cells. The Dmt-Tic (**3**−**5**) and Dmt-D-Tic (**6**−**10**) analogues generally exhibited high binding affinity (K_i = 0.11−15 nM) at both μ and δ opioid receptors, but distinct subtype selectivity. Only Dmt-D-Tic-D-Phe(*p*-Cl or *p*-F) analogues **9** and **10** resulted in lower affinities (K_i = 130 and

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Table 1. Structure of Dmt-L(or D)-Tic Analogues

		H-Dmt[1]-Xxx[2]-Yyy[3]-Z[4]		
analogue	aLOGPs[a]	Xxx[2]	Yyy[3]	Z[4]
3	4.30	Tic	phe	compound 1
4	4.91	Tic	Phe(*p*-Cl)	compound 1
5[b]	4.30	Tic	D-Phe(*p*-Cl)	compound 1
6	4.91	D-Tic	phe	compound 1
7	4.91	D-Tic	Phe(*p*-Cl)	compound 1
8	4.41	D-Tic	Phe(*p*-F)	compound 1
9	4.91	D-Tic	D-Phe(*p*-Cl)	compound 1
10	4.85	D-Tic	D-Phe(*p*-Cl)	compound 2

[a]http://www.vcclab.org/lab/alogps/. [b]See ref 8.

250 nM, respectively) at the δ receptor. All analogues were tested for their efficacy in GTP-γ-S assay and for antagonist (or agonist) activity in functional assays, respectively, and both assay results were correlated with each other. As predicted, the functional assays resulted in dissimilar prototypes of functional activities for the δ opioid receptor depending upon the chiralities of positions 2 and 3. All the series of analogues showed a broad range of agonist activities but no antagonist activities at the μ opioid receptor. In this series, analogue **4** exhibited the best δ opioid antagonism (K_e = 0.79 ± 0.6 nM)/ moderate μ opioid agonism (IC_{50} = 350 ± 90 nM), which can be an interesting clinical analgesic on the basis of its low propensity to cause tolerance and dependence during chronic use.[9−11]

The H-Dmt-L(or D)-Tic analogues were prepared by stepwise solution-phase peptide syntheses starting from commercially available *N*-phenyl-*N*-piperidin-4-yl-propionamide (for analogues **3**−**9**) or 4-((1*H*-1,2,4-triazol-1-yl)-methyl)-4-cyclohexylpiperidine (for analogue **10**) using N^{α}-Boc-strategy in high yields (overall yields in up-to 8 steps > 45%) (Scheme 1).[12,13] Each coupling reaction used 1.1 equiv BOP/1.1 equiv HOBt/2.5 equiv NMM for 2−3 h at room temperature. During the chain elongations, pure peptide intermediates were obtained by simple precipitation from appropriate organic solvents, usually diethyl ether. The syntheses proceeded very well resulting in high purity of peptides. The crude peptides showed more than 90% purity and could be further purified by prep. RP-HPLC using a gradient system composed of acetonitrile and a 0.1% aqueous TFA solution. The structures were confirmed by HR-MS spectroscopy and ^{1}H NMR spectroscopy (see Supporting Information).

In vitro bioassays were carried out as previously described.[14] Opioid binding affinities at the human δ opioid receptor (hDOR) and the rat μ opioid receptor (rMOR) were determined by competition analyses against [^{3}H]DPDPE (δ) and [^{3}H]DAMGO (μ) using membrane preparations from transfected HN9.10 cells that constitutively express the respective receptors. Opioid agonist efficacy was examined by monitoring [^{35}S]GTP-γ-S binding. Functional assays to evaluate their opioid agonist activities were performed using the stimulated isolated mouse vas deferens (MVD, δ) and guinea pig ileum (GPI, μ) bioassays. In general, the functional assay results correlated with those from the [^{35}S]GTP-γ-S binding assay.

In the competition binding assay, all the Dmt-L(or D)Tic analogues except **9** and **10** showed high binding affinities for both μ and δ receptors, but with distinct selectivities depending upon the configuration of Tic[3] residue (Table 2). Ligands **3**−**5**,

Table 2. Binding Affinities of Dmt-L(or D)Tic Analogues at μ and δ Opioid Receptors

	hDOR[a] [^{3}H]DPDPE[b]		rMOR[a] [^{3}H]DAMGO[c]		
no.	log IC_{50}[d]	K_i (nM)[e]	log IC_{50}[d]	K_i (nM)[e]	$K_i\mu/K_i\delta$
3	−9.37 ± 0.11	0.20	−7.70 ± 0.12	9.5	48
4	−9.17 ± 0.10	0.43	−7.49 ± 0.13	15	35
5[f]		0.38		22	58
6	−8.80 ± 0.11	0.76	−9.02 ± 0.06	0.45	0.59
7	−7.93 ± 0.07	5.5	−7.54 ± 0.10	14	2.5
8	−7.69 ± 0.15	9.3	−8.39 ± 0.08	1.9	0.20
9	−6.56 ± 0.08	130	−7.60 ± 0.08	12	0.09
10	−6.28 ± 0.10	250	−7.87 ± 0.04	6.4	0.026
Dmt-Tic-NH_2[g]		1.2		280	230
Dmt-D-Tic-NH_2[g]		57		3.8	0.067
Dmt-Tic-Phe-Phe -NH_2[h]		0.12		1.2	10

[a]Competition analyses were carried out using membrane preparations from transfected HN9.10 cells that constitutively expressed the respective receptor types. [b]K_d = 0.50 ± 0.10 nM. [c]K_d = 0.85 ± 0.20 nM. [d]Logarithmic values determined from the nonlinear regression analysis of data collected from at least three independent experiments. [e]Antilogarithmic value of the respective IC_{50}. [f]See ref 7. [g]See ref 1. [h]See refs 9 and 10.

which have an L-Tic[2] residue, bind to the δ opioid receptor better than the μ opioid receptor by 35−58 fold. The change of configuration from L- to D-Tic[2] abolished high δ opioid affinity while simultaneously increasing μ affinity to yield ligands with either reduced δ selectivity or the acquisition of μ selectivity, similar to changes in affinity and selectivity observed with isomeric modifications in Dmt-Tic analogues. It is well-known that the chirality of Tic residue plays an important role in the activity of Dmt-Tic moiety contained tripeptides as well as in the TIP(P′) peptides.[1,15] Our result supports the fact that, while Dmt-Tic represents the δ opioid message domain, Dmt-D-Tic better defines a μ opioid message domain.[1,7,15]

In ligands **3**−**5**, halogenations of the aromatic ring in the Phe[3] residue and substituting the L-Phe(*p*-Cl)[3] residue with D-residue did not affect binding affinities and selectivities at either receptor. Therefore, it is considered that the Dmt-Tic moiety plays a dominate role in the affinity, and the third amino acid residue does not affect it critically. This result is consistent with earlier reports that *cis*−*trans* equiliblium of the peptide backbone between positions 1 and 2 in Tyr-Tic-(L- or D-)Phe depends mostly on the configurations of Tyr and Tic residues forming the peptide bond, but not the Phe residue following the sequence.[16]

In contrast, in ligands **7** and **8**, which contain the Dmt-D-Tic moiety, *p*-chloro or *p*-fluoro substitutions on the aromatic ring of the Phe[3] residue led to reduced bining affinities at both μ and δ receptors. In previous studies, we showed that halogenation of the aromatic ring of a Phe[4] residue in enkephalin-like ligands enhanced activities at both opioid receptors.[13] Our current SAR result was different than the known effect of halogenation on enkephalin analogues. It is clear that this conflicting result is due to the absence of the Gly[3] residue, which can separate the D-Tic[2] residue from the aromatic ring of a Phe residue.

Whereas most ligands showed good binding affinities at the δ opioid receptor in the subnanomolar to the nanomolar range, ligand **9**, in which the configuration of the third amino acid residue was the D-form, lost its binding affinitiy (K_i = 130 nM) 24-fold. However, its binding affinity at the μ receptor was

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Table 3. Functional activities of Dmt-L(or D)Tic analogues

	[^{35}S]GTP-γ-S binding						MVD(δ)		GPI(μ)	
	hDOR[a]			rMOR[a]			agonist	antagonist	agonist	antagonist
no.	log EC_{50}[b]	EC_{50} (nM)[c]	E_{max} (%)[d]	log EC_{50}[b]	EC_{50} (nM)[c]	E_{max} (%)[d]	IC_{50} (nM)[e]	K_e (nM)[f]	IC_{50} (nM)[e]	K_e (nM)[f]
3		nr			nr		20%	5.0 ± 3	740 ± 220	
4		nr			nr		1.6%	0.79 ± 0.6	350 ± 90	
5[g]		0.48	18		6.2	16	74 ± 14		0%	nr
6	−8.09 ± 0.07	8.1	128	−9.04 ± 0.28	0.90	40	42+6		82 ± 9	
7		nr			nr		14%	150 ± 20	27%	nr
8	−7.84 ± 0.14	15	47	−8.35 ± 0.17	4.5	82	86 ± 29		720 ± 210	
9		nr			nr		0%	nr	4%	nr
10		nr			nr		810 ± 140	nr	6%	nr
Dmt-Tic-NH_2[h]								42	>10 μM	
Dmt-Tic-Phe-Phe-NH_2[i]								0.21 ± 0.04	18 ± 2	

[a]Expressed in CHO cells. [b]Logarithmic values determined from the nonlinear regression analysis of data collected from at least three independent experiments. [c]Antilogarithmic value of the respective EC_{50} values. [d]Net total bound/basal binding ×100 ± SEM. [e]Concentration at 50% inhibition of muscle contraction at electrically stimulated isolated tissues or % inhibition of contraction height at 1 μM. [f]Antagonist. [g]See ref 7. [h]See ref 1. [i]See refs 9 and 10. nr: no response.

maintained in a similar range (K_i = 12 nM) to those of the other ligands. Apparently replacement of the piperidinyl ring in position 4 of ligand **9** with the triazoly containing ring in ligand **10** did not affect the binding affinities but slightly changed the selectivity. On the basis of these SAR results, it is clear that, in this series of analogues, the configurations of Tic[2] and Phe[3] play an important role in determining their binding affinities and receptor selectivities.

As predicted, Dmt-Tic analogues **3** and **4** did not show agonist stimulation at both δ and μ receptors in the [^{35}S]GTP-γ-S binding assay but exhibited strong δ antagonist (K_e = 5.0 ± 3 nM)/weak μ agonist (IC_{50} = 740 ± 220 nM) activities in MVD/GPI assays, respectively (Table 3). As mentioned earlier, surprisingly, the ligand **5**, in which the chirality of third amino acid residue was changed from L- to D-, exhibited δ agonist activity instead of antagonist activity in the [^{35}S]GTP-γ-S (EC_{50} = 0.48 nM, E_{max} = 18%) and the MVD (IC_{50} = 74 ± 14 nM) assays. According to a previous report, the distance between the Dmt-Tic and the third aromatic nucleus is an important criterion in converting Dmt-Tic from a highly potent δ antagonist into a potent δ agonist or into ligands with mixed δ and μ opoioid properties.[3] This fact can help us to elucidate how ligand **5** has a moderate partial δ agonist activity instead, which is inconsistent with other Dmt-Tic analogues containing a D-amino acid residue, for example, D-Ala, D-Asp, and D-Gln, in position 3.[14] Even with moderate binding affinity (K_i = 22 nM) and efficacy (EC_{50} = 6.2 nM) at the μ opioid receptor, ligand **5** did not show agonist activity in the GPI assay. This may be the result of the conflicting tendencies of the Dmt-Tic message domain to impart δ antagonism. It is possible that, for the same reason, the ligand showed partial agonist activity with low E_{max} (18%) but with high affinity (K_i = 0.38 nM).

It was shown that the Tic[2] residue in Dmt-contained peptides plays an important role in their biological activities for the δ opioid receptor, and thus a significant loss in the receptor binding affinity and bioactivity occurred when D-Tic is utilized.[17] In most cases, Dmt-D-Tic-Phe analogues behave like Tyr-D-Xaa-Phe peptides and have μ agonism. As predicted, Dmt-D-Tic analogues **6** and **8** showed higher binding affinity (K_i = 0.45 and 1.9 nM, respectively) and agonist efficacy (EC_{50} = 0.90 and 4.5 nM, respectively), but relatively lower agonist activity (IC_{50} = 82 and 720 nM) at the μ receptor. However, ligand **7**, as observed in the binding assay, exhibited a different biological profile from ligands **6** and **8** and the ligand was not an agonist for the δ and μ receptors, but a weak antagonist (K_e = 150 nM) for the δ receptor. It was shown that *p*-chloro substitution of the aromatic ring of Phe[3] residue in Dmt-Tic analogues did not change its biological activity, and thus, ligands **3** and **4** showed very similar biological profiles. Therefore, it is clear that Dmt-D-Tic analogues showed distinct SAR compared to Dmt-Tic analogues.

As published before, the Dmt[1]-D-Tic[2]-Gly[3]-Phe(*p*-Cl)[4] analogue did not show δ opioid antagonist activity, but a potent agonist activity in the MVD assay (IC_{50} = 0.21 nM, μ/δ = 23) unlike ligand **7**, which includes a Phe(*p*-Cl)[3] residue and exhibits weak antagonist activity (IC_{50} = 170 nM). Again, this functional assay result confirms that insertion of a Gly residue into position 3 of Dmt-D-Tic analogues can distinguish a topographical structure from the other ligands.

On the basis of the binding affinities of ligands **9** and **10**, the consecutive change of configuration (L,L to D,D) was not tolerated for the δ receptor, and thus, the ligands **9** and **10** have reduced binding affinities at the δ receptor. While the ligands showed moderate binding affinities (K_i = 12 and 6.4 nM) and selectivity for the μ receptor, no functional activity was observed in the [^{35}S]GTP-γ-S assay and the GPI assay, and instead, low δ opioid agonist function (IC_{50} = 810 ± 140 nM) was observed in the MVD assay. Overall, the opioid agonist activities (IC_{50}) in the GPI assay were relatively less than the one in the MVD assay, and the discrepancy seems to be the result caused by conflict between the Dmt-Tic (or Dmt-D-Tic) message and the analogues.[18]

In conclusion, we observed that the chiralities of Tic and Phe residues in positions 2 and 3 have a large influence on the binding affinity and efficacy profile of Dmt-L-(or D-)Tic analogues. Whereas Dmt-Tic-Phe analogues are δ receptor selective antagonists and Dmt-Tic-D-Phe analogues are δ receptor selective agonists, Dmt-D-Tic-Phe and Dmt-D-Tic-Gly analogues are nonselective δ/μ agonists, except for the analogue that includes a Phe(*p*-Cl)[3] residue and shows δ antagonist activity. In contrast, no functional activity except low δ agonist activity was observed in Dmt-D-Tic-D-Phe analogues.

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■ ASSOCIATED CONTENT

Supporting Information

Analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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■ ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazole-1-yloxy)-tris-(dimethylamino)-phosphonium hexafluorophosphate; CHO, Chinese hamster ovary; DMF, *N*,*N*-dimethylformamide; hDOR, human δ opioid receptor; DPDPE, c[D-Pen^2,D-Pen^5]-enkephalin; DAMGO, [D-Ala^2,NMePhe^4,Gly^5-ol]enkephalin; Dmt, 2,6-dimethyltyrosine; GPI, guinea pig isolated ileum; HOBt, 1-hydroxybenzotriazole; rMOR, rat μ opioid receptor; MVD, mouse vas deferens; NMM, *N*-methylmorpholine; RP-HPLC, reverse phase high performance liquid chromatography; SAR, structure−activity relationships; TFA, trifluoroacetic acid

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