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[Dmt¹]DALDA analogues modified with tyrosine analogues at position 1

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Abstract

Analogues of [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂; Dmt = 2',6'-dimethyltyrosine), a potent μ opioid agonist peptide with mitochondria-targeted antioxidant activity were prepared by replacing Dmt with various 2',6'-dialkylated Tyr analogues, including 2',4',6'-trimethyltyrosine (Tmt), 2'-ethyl-6'-methyltyrosine (Emt), 2'-isopropyl-6'-methyltyrosine (Imt) and 2',6'-diethyltyrosine (Det). All compounds were selective μ opioid agonists and the Tmt¹-, Emt¹ and Det¹-analogues showed subnanomolar μ opioid receptor binding affinities. The Tmt¹- and Emt¹-analogues showed improved antioxidant activity compared to the Dmt¹-parent peptide in the DPPH radical-scavenging capacity assay, and thus are of interest as drug candidates for neuropathic pain treatment.

Keywords

[Dmt¹]DALDA; Bifunctional opioid/antioxidant peptides; Tyrosine analogues; Opioid activity profiles; Neuropathic pain

The dermorphin-derived tetrapeptide DALDA (H-Tyr-D-Arg-Phe-Lys-NH₂) (**6**) is a μ opioid receptor agonist with low nanomolar binding affinity (K_i^{μ} = 1.69 nM) and high μ receptor selectivity (Tables 1 and 2).¹ Replacement of Tyr¹ in DALDA with 2',6'-dimethyltyrosine (Dmt) resulted in a μ opioid agonist, [Dmt¹] DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂) (**5**) with subnanomolar binding affinity and with still high preference for μ receptors over δ and κ opioid receptors.² The potency-enhancing effect of Dmt substitution for Tyr¹ in opioid peptides was first demonstrated by Hansen et al.³ and has found widespread use in the design of opioid peptides analogues with increased agonist or antagonist activity at opioid receptors. For example, the mixed μ agonist/ δ antagonist DIPP-NH₂ (H-Dmt-Tic-Phe-Phe-

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Supplementary data

Supplementary data (full experimental procedure for synthesis of peptides and chemical and biological characterization of peptides) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2016.06.003>.

NH₂; Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) showed 65- and 25-fold increased binding affinity at the μ and δ receptors, respectively, as compared to TIPP-NH₂ (H-Tyr-Tic-Phe-Phe-NH₂).⁴ A recently determined crystal structure of DIPP-NH₂ in complex with the δ opioid receptor showed that the two methyl groups of Dmt interact with hydrophobic receptor residues.⁵ These hydrophobic interactions strengthen binding and likely are responsible for the potency increases seen with all Dmt¹-containing opioid peptides.

[Dmt¹]DALDA showed high antinociceptive potency in the rat and mouse tail-flick assays. In comparison with morphine, it was 3000-fold more potent given intrathecally (i.t.)⁶ and 40–220-fold more potent given subcutaneously (s.c.)^{7,8} in these acute pain models, indicating that it is capable of crossing the blood-brain barrier (BBB). Furthermore, the duration of the antinociceptive effect of [Dmt¹]DALDA was 4 times longer than that of morphine^{6,9} and its demonstrated resistance to enzymatic degradation and slow clearance indicated good druglike properties.⁹

[Dmt¹]DALDA has been shown to be taken up into various types of cells, including neuronal cells and to selectively target the inner mitochondrial membrane (IMM).^{10,11} Its ability to cross cellular membranes and to distribute to the IMM is due to the structural motif of its amino acid sequence of alternating aromatic and basic residues.¹¹ The *N*-terminal Dmt residue of [Dmt¹]DALDA has antioxidant activity due to the two electron-donating methyl groups on its phenol moiety, similar to the methylated phenol structure contained in vitamin E. [Dmt¹]DALDA has been shown to be an effective scavenger of oxyradicals.¹¹

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide hydroxyl radical and peroxynitrite, play an important role in the development and maintenance of neuropathic pain^{12,13} and complex regional pain syndrome-type I (CRPS-I).¹⁴ ROS are produced by the mitochondrial electron transport chain and by the enzymes xanthine oxidase and NADPH oxidase. Because of its bifunctional activity profile as μ opioid agonist and mitochondria-targeted antioxidant, [Dmt¹]DALDA in comparison with morphine was examined in the spinal nerve ligation (SNL) rat model of neuropathic pain with s.c. administration.¹⁵ At doses that were equianalgesic in naive animals, [Dmt¹]DALDA was more effective than morphine in increasing the thermal pain threshold in thermal hyperalgesia in the SNL model. The superior antinociceptive effect of [Dmt¹]DALDA can be explained by an additive or synergistic effect of its μ opioid agonist and antioxidant activity. In a study using the chronic post ischemia pain (CPIP) rat model of CRPS-I¹⁶ it was shown that μ opioid receptor activation and antioxidant administration indeed produced a synergistic effect in reversing mechanical allodynia.¹⁷ In agreement with this finding, [Dmt¹]DALDA was 15 times more potent than morphine in reversing mechano-allodynia in the CPIP model.¹⁷ Taken together, these results indicate that bifunctional compounds with μ opioid agonist/antioxidant activity have therapeutic potential for the treatment of neuropathic pain and CRPS-I.

Recently, we reported analogues of [Dmt¹]DALDA in which the Phe³ residue had been replaced by various 2',6'-dialkylated Phe analogues, including 2',6'-dimethylphenylalanine (Dmp), 2',4', 6'-trimethylphenylalanine (Tmp), 2'-isopropyl-6'-methylphenylalanine (Imp)

and 2'-ethyl-6'-methylphenylalanine (Emp).¹⁸ In comparison with the [Dmt¹]DALDA parent, the Dmp³- and Emp³-analogues showed enhanced μ receptor binding affinity, and while the Dmp³- and Tmp³-analogues retained high μ receptor selectivity, the Imp³- and Emp³-analogues displayed little preference for μ receptors over κ receptors. These interesting results prompted a study on the synthesis and pharmacological evaluation of [Dmt¹]DALDA analogues containing various alkyl substituents on the phenol ring of Tyr¹: 2',3',6'-trimethyltyrosine (Tmt), 2'-ethyl-6'-methyltyrosine (Emt), 2'-isopropyl-6'-methyltyrosine (Imt) and 2',6'-diethyltyrosine (Det) (Fig. 1).

The unnatural amino acids Tmt, Emt, Imt and Det were prepared by a previously reported synthetic method based on [Ru(1,5-(COD))(R,R-DIPAMP)BF₄]-mediated asymmetric catalytic hydrogenation of the corresponding acetamidoacrylates.¹⁹ Peptides were synthesized by the solid-phase technique according to a published protocol.¹⁸

Binding affinities for μ , δ and κ opioid receptors were determined by displacement of receptor-selective radioligands from rat or guinea pig brain membranes, as previously reported.² For the determination of opioid agonist potencies the functional assays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) (μ receptor-representative)²⁰ and the mouse vas deferens (MVD) (δ receptor-representative)²¹ were used.

In the opioid receptor-binding assays (Table 1), H-Tmt-D-Arg-Phe-Lys-NH₂ (**1**) and H-Emt-D-Arg-Phe-Lys-NH₂ (**2**) showed high, subnanomolar μ receptor binding affinities that were about 2–3-fold lower than that of [Dmt¹]DALDA (**5**), but 4–5-fold higher than that of the DALDA parent (**6**). This indicates that, as in the case of [Dmt¹]DALDA, the alkyl substituents on the phenol ring of Tmt and Emt engage in hydrophobic interactions with receptor residues. With the Det¹-analogue (**4**) a further 2-fold decrease in μ receptor binding affinity was observed and it thus turned out to be about 6 times less potent than [Dmt¹]DALDA (**5**). The moderately decreased μ receptor binding affinities seen with compounds **1**, **2** and **4** as compared to **5** likely are due to some slight steric interference at the receptor binding site. The more drastic, 30-fold μ receptor binding affinity decrease seen with the Imt¹-analogue (**3**) may be due to the restricted rotational flexibility of the isopropyl substituent on the phenol ring of Imt which may cause more severe steric interference. A molecular dynamic stimulation had shown that in an analogously substituted phenylalanine analogue, 2'-isopropyl-6'-methylphenylalanine (Imp), the rotational flexibility of the isopropyl substituent was severely restricted, with only one conformational state being occupied in the course of the simulation performed at 300 K with a duration of 1 ns.¹⁸ In this case, the restricted rotational flexibility of the isopropyl residue in the Imp³-analogue of [Dmt¹]DALDA did not affect μ receptor binding affinity, indicating that this rotational constraint was tolerated at the Phe³; however, it had a major effect on the receptor selectivity due to a 100-fold increase in κ opioid receptor binding affinity.

All compounds showed very weak δ opioid receptor binding affinities with K_i^δ s ranging from 570 to 4670 nM (Table 1), as well as weak κ opioid receptor binding affinities (K_i^κ s=60 – 1470nM). Thus, they are highly μ receptor-selective compounds, as is the case

with [Dmt¹]DALDA. These results indicate that variation of the substituents in the 2',6'-positions of Tyr¹ had no significant effect on the opioid receptor selectivity profile.

The rank order of compound agonist potencies determined in the functional GPI assay was the same as that observed in the μ receptor binding assay (Table 2). Analogues **1** and **2** were 14- and 18-fold more potent, respectively, than the DALDA parent (**6**). Again, the Imt¹-analogue (**3**) was the weakest agonist of the series with a potency 1.6-fold lower than that of **6**. The same rank order of the compounds' agonist potency was observed in the δ receptor-representative MVD assay, with compound **3** again showing the weakest δ agonist potency. In agreement with the receptor binding data, the IC₅₀ values determined in the MVD assay were much higher than those seen in the GPI assay, thus confirming the μ receptor selectivity of all compounds.

The parent peptide [Dmt¹]DALDA (**5**) was shown to have quite good antinociceptive potency in animal models of neuropathic pain as a consequence of its combined μ opioid agonist activity and antioxidant properties.^{15,17} Theoretical²² and experimental²³ studies on the antioxidant abilities of phenols indicate that phenols with an increased number of electron-donating groups or with stronger electron-donating groups have stronger antioxidant ability. In comparison with **5**, the Tmt¹- and Emt¹-analogues (**1** and **2**) are expected to have higher antioxidant activity, because Tmt has three electron-donating methyl groups and Emt contains an ethyl group which is a stronger electron-donating group than the corresponding methyl group in Dmt. The expected improved antioxidant abilities of **1** and **2** were confirmed by determining their DPPH radical-scavenging activity,²⁴ according a published experimental protocol.²⁵ A DPPH concentration of 0.085 mM and peptide solutions of 0.13 mg/ml were used. [Tmt¹]DALDA (**1**), [Emt¹]DALDA (**2**) and [Dmt¹]DALDA (**5**) showed a DPPH scavenging effect of 34.58%, 33.51% and 31.64%, respectively. A higher scavenging effect value corresponds to higher antioxidant activity.

In conclusion, all compounds showed high preference for μ over δ and κ opioid receptors and the μ receptor binding affinities of two of them, H-Tmt-D-Arg-Phe-Lys-NH₂ (**1**) and H-Emt-D-Arg-Phe-Lys-NH₂ (**2**) were still in the subnanomolar range and only slightly lower than that of [Dmt¹]DALDA (**5**). In comparison with **5**, the Tmt¹- and Emt¹-analogues (**1** and **2**) showed higher antioxidant activity. Since μ opioid receptor activation and antioxidant activity have been shown to produce a synergistic effect in alleviating mechanical allodynia in the CPIP model of CRPS-I¹⁷, these compounds are still of interest as drug candidates for neuropathic pain treatment, despite their 2–3-fold lower μ receptor binding affinity as compared to [Dmt¹]DALDA. The use of a single bifunctional compound with combined μ opioid agonist and antioxidant activity is preferable to a two-component cocktail of a μ opioid and antioxidant because of the more predictable pharmacokinetic and pharmacodynamic outcome resulting from the administration of a single compound.²⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BBB	blood–brain–barrier
Boc	tert-butyloxycarbonyl
CPIP	chronic postischemia pain
CRPS-I	complex regional pain syndrome-type I
DALDA	H-Tyr-D-Arg-Phe-Lys-NH ₂
DAMGO	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol
Det	2',6'-diethyltyrosine
DIEA	<i>N,N</i> -diisopropylethylamine
DIPP-NH₂	H-Dmt-Tic-Phe-Phe-NH ₂
Dmp	2',6'-dimethylphenylalanine
Dmt	2',6'-dimethyltyrosine
[Dmt¹]DALDA	H-Dmt-D-Arg-Phe-Lys-NH ₂
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
EDT	1,2-ethanediol
Emp	2'-ethyl-6'-methylphenylalanine
Emt	2'-ethyl-6'-methyltyrosine
Fmoc	9-fluorenylmethoxycarbonyl
GPI	guinea pig ileum
HOBt	1-hydroxybenzotriazole
IMM	inner mitochondrial membrane
Imp	2'-isopropyl-6'-methylphenylalanine
Imt	2'-isopropyl-6'-methyltyrosine
i.t	intrathecal

MVD	mouse vas deferens
Pbf	(2,3-dihydro-2,2,4,6,7-pentamethyl-5-benzofuranyl)sulfonyl
PyBOP	(benzotriazol-1-yl-oxy)tris (pyrrolidino)phosphonium hexafluorophosphate
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP-HPLC	reversed-phase high performance liquid chromatography
s.c.	subcutaneous
SNL	spinal nerve ligation
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TIPP-NH₂	H-Tyr-Tic-Phe-Phe-NH ₂
TLC	thin layer chromatography
Tmp	2',4',6'-trimethylphenylalanine
Tmt	2',3',6'-trimethyltyrosine
U69	593, (5 <i>R</i> ,7 <i>S</i> ,8 <i>S</i> -(<i>-</i>)- <i>N</i> -methyl- <i>N</i> -[7-(1-pyrrolidiny)]-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide

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Figure 1.
Structural formulas of Dmt, Tmt, Emt, Imt and Det.

Table 1

Opioid receptor binding affinities of DALDA analogues

No.	Compound	Opioid receptor binding ^a			Potency ratio $\mu/\delta/\kappa$
		K_i^μ [nM]	K_i^δ [nM]	K_i^κ [nM]	
1	H-Tmt-D-Arg-Phe-Lys-NH ₂	0.458 ± 0.010	2900 ± 320	381 ± 8	1/6330/832
2	H-Emt-D-Arg-Phe-Lys-NH ₂	0.367 ± 0.035	573 ± 141	58.1 ± 6.4	1/1560/158
3	H-Imt-D-Arg-Phe-Lys-NH ₂	4.29 ± 0.99	4670 ± 910	472 ± 29	1/1090/110
4	H-Det-D-Arg-Phe-Lys-NH ₂	0.875 ± 0.086	2360 ± 520	210 ± 50	1/2700/240
5	H-Dmt-D-Arg-Phe-Lys-NH ₂ ([Dmt ¹] DALDA)	0.143 ± 0.015	2100 ± 310	22.3 ± 4.2	1/14700/156
6	H-Tyr-D-Arg-Phe-Lys-NH ₂ (DALDA)	1.69 ± 0.015	19200 ± 2000	4230 ± 360	1/11400/2500

^aMean of 3–4 determinations ± SEM.

Table 2

GPI and MVD assay of DALDA analogues

No.	Compound	GPI	MVD
		IC ₅₀ [nM] ^a	IC ₅₀ [nM] ^a
1	H-Tmt-D-Arg-Phe-Lys-NH ₂	18.4 ± 1.1	290 ± 19
2	H-Emt-D-Arg-Phe-Lys-NH ₂	14.0 ± 1.4	137 ± 8
3	H-Imt-D-Arg-Phe-Lys-NH ₂	410 ± 49	7170 ± 2210
4	H-Det-D-Arg-Phe-Lys-NH ₂	55.7 ± 3.8	755 ± 80
5	H-Dmt-D-Arg-Phe-Lys-NH ₂ ([Dmt ¹] DALDA)	1.41 ± 0.29	23.1 ± 2.0
6	H-Tyr-D-Arg-Phe-Lys-NH ₂ (DALDA)	254 ± 27	781 ± 146

^a Mean of 3–4 determinations ± SEM.