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N-terminal guanidinylation of TIPP (Tyr-Tic-Phe-Phe) peptides results in major changes of the opioid activity profile

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Abstract

Derivatives of peptides of the TIPP (Tyr-Tic-Phe-Phe; Tic = 1,2,3,4- tetrahydroisoquinoline-3- carboxylic acid) family containing a guanidino (Guan) function in place of the N-terminal amino group were synthesized in an effort to improve their blood-brain barrier permeability. Unexpectedly, N-terminal amidination significantly altered the in vitro opioid activity profiles. Guan-analogues of TIPP-related opioid antagonists showed partial agonist or mixed partial agonist/ μ partial agonist activity. Guanidinylation of the mixed μ agonist/ antagonists H-Dmt-Tic-Phe-Phe-NH₂ (DIPP-NH₂) and H-Dmt-Tic [CH₂NH]Phe-Phe-NH₂ (DIPP-NH₂[]) converted them to mixed μ agonist/ agonists. A docking study revealed distinct positioning of DIPP-NH₂ and Guan-DIPP-NH₂ in the receptor binding site. Lys³-analogues of DIPP-NH₂ and DIPP-NH₂[] (guanidinylated or non-guanidinylated) turned out to be mixed μ / agonists with antagonist-, partial agonist- or full agonist activity. Compounds with some of the observed mixed opioid activity profiles have therapeutic potential as analgesics with reduced side effects or for treatment of cocaine addiction.

Keywords

Peptide synthesis; guanidinylated opioid peptides; opioid antagonists; partial opioid agonists; μ opioid agonist/ opioid antagonists; μ opioid agonist/ opioid agonists

The tetrapeptides H-Tyr-Tic-Phe-Phe-OH (TIPP) (**1a**), H-Dmt-Tic-Phe-Phe-OH (DIPP) (**2a**) and H-Tyr-Tic [CH₂NH]Phe-Phe-OH (TIPP[]) (**3a**) are highly selective opioid antagonists with low nanomolar or subnanomolar opioid receptor binding affinity.^{1–3} The TIPP-derived tetrapeptide amides H-Dmt-Tic-Phe-Phe-NH₂ (DIPP-NH₂) (**4a**) and H-Dmt-Tic [CH₂NH]Phe-Phe-NH₂ (DIPP-NH₂[]) (**5a**) act as agonists at the μ opioid receptor and as antagonists at the δ receptor. On the basis of a well established pharmacological rationale,^{4,5} compounds with such a mixed μ agonist/ antagonist profile are expected to be analgesics with low propensity to produce analgesic tolerance and physical dependence.

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Supplementary data

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Indeed, DIPP-NH₂ [] given i.c.v. produced a potent analgesic effect in the rat tail flick test and upon chronic administration induced less analgesic tolerance than morphine and no physical dependence.⁶ Both opioid antagonists and mixed μ agonist/ antagonists of the TIPP peptide family proved to be valuable tools in opioid receptor research and proof-of-concept studies in the opioid field. However, TIPP peptides do not effectively cross the BBB.

Cationization of proteins and peptides by guanidine (Guan) addition has been shown to improve metabolic stability and BBB permeability.^{7,8} N-terminal amidination of a μ -selective dermorphin-derived tetrapeptide did not result in a significant change in μ receptor binding affinity and high selectivity for μ vs. δ and κ receptors was retained.⁹ The resulting compound produced centrally mediated analgesia with oral and s.c. administration, indicating its ability to cross the BBB. N-terminally amidinated endorphin-1 analogues displayed somewhat reduced μ receptor binding affinity as compared to their respective non-amidinated parent peptides, were still μ receptor-selective and showed enhanced BBB permeability.¹⁰

Here we describe the syntheses and in vitro opioid activity profiles of the N-terminally amidinated TIPP antagonists **1a**, **2a** and **3a**: Guan-Tyr-Tic-Phe-Phe-OH (**1**), Guan-Dmt-Tic-Phe-Phe-OH (**2**) and Guan-Tyr-Tic-[CH₂NH]Phe-Phe-OH (**3**), and of the mixed μ agonist/antagonists **4a** and **5a**: Guan-Dmt-Tic-Phe-Phe-NH₂ (**4**) and Guan-Dmt-Tic-[CH₂NH]-Phe-Phe-NH₂ (**5**). The novel Lys³-containing peptides H-Dmt-Tic-Lys-Phe-NH₂ (**6a**) and H-Dmt-Tic-[CH₂NH]Lys-Phe-NH₂ (**7a**), and their respective guanidinylated analogues Guan-Dmt-Tic-Lys-Phe-NH₂ (**6**) and Guan-Dmt-Tic-[CH₂NH]Lys-Phe-NH₂ (**7**) were also synthesized and pharmacologically characterized. Finally, we describe the synthesis and in vitro opioid activity profile of the guanidinylated dipeptide opioid antagonist H-Dmt-Tic-OH¹¹ (**8a**): Guan-Dmt-Tic-OH (**8**).

Peptides **1–7** were synthesized by the manual solid-phase method using a Boc-Phe resin for peptides **1–3** and a *p*-methylbenzhydrylamine resin for peptides **4**, **5**, **6**, **6a**, **7** and **7a** with Boc-protection and DIC/Cl-HOBt as coupling agents. To introduce the reduced peptide bond between the Tic² and Phe³ or Tic² and Lys³ residues in peptides **3**, **5**, **7** and **7a**, a reductive alkylation reaction¹² between 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde¹³ and the α -amino group of the resin-bound H-Phe-Phe- or H-Lys-Phe dipeptide segments was performed. Amidination on the resin was performed using the reagent *N,N*-bis-(2-chloro-benzoyloxycarbonyl)-1*H*-1-pyrazole-1-carboxamide.¹⁴ Peptides were cleaved from the resin by HF/anisole treatment. The guanidinylated dipeptide Guan-Dmt-Tic-OH (**8**) was prepared in solution by coupling Boc-Dmt-OH with H-Tic-OMe using HBTU as coupling agent. The reagent 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine¹⁵ was used for N-terminal guanidinylation. Subsequent NaOH hydrolysis of the methyl ester and Boc deprotection with TFA afforded the target product. Crude products were purified by reversed-phase HPLC and their purity (>98%) and structural identity were established by TLC, analytical HPLC and ES-MS.

Binding affinities (K_i values) for μ and δ opioid receptors were determined by displacing, respectively, [³H]DAMGO and [³H]DSLET from rat brain membrane binding sites, and opioid receptor binding affinities were measured by displacement of [³H]U69,593 from guinea pig brain membrane binding sites, as described.¹⁶ Opioid agonist potencies (IC₅₀ values) or antagonist activities (K_e values) were determined in the mouse vas deferens (MVD) assay (δ receptor-representative) or in the guinea pig ileum (GPI) assay (μ and κ receptor-representative) using previously described protocols.¹⁶

Guanidinylation of the antagonist TIPP (**1a**) resulted in a compound (**1**) with receptor binding affinity ($K_i = 2.29$ nM) similar to that of the TIPP parent ($K_i = 1.22$ nM) (Table 1). In comparison with the antagonists DIPP (**2a**) and TIPP[] (**3a**), the respective guanidylated peptides Guan-Dmt-Tic-Phe-Phe-OH (**2**) and Guan-Tyr-Tic [CH₂NH]Phe-Phe-OH (**3**) retained similar subnanomolar receptor binding affinities ($K_i = 0.146$ nM and 0.968 nM, respectively). Like their parent peptides, the guanidylated peptides **1**, **2** and **3** showed high receptor binding selectivity with weak binding affinities for μ opioid receptors ($K_i^\mu = 126 - 875$ nM) and very weak affinity for κ receptors ($K_i > 2000$ nM). Whereas peptides **1a**, **2a** and **3a** were potent antagonists in the MVD assay with K_e values in the $0.2 - 4.8$ nM range, the guanidylated peptides **1**, **2** and **3** showed partial agonist behavior (Table 2). For peptide **2** an IC_{35} of 1.57 nM could be determined based on 70% maximal inhibition of the electrically evoked contractions of the vas produced by this compound. Peptides **1** and **3** showed lower maximal inhibitions to the extent of 33% and 50%, respectively, which did not permit the determination of accurate IC values.

Guanidinylation of the mixed μ agonist/ antagonist H-Dmt-Tic-Phe-Phe-NH₂ (**4a**) had unexpected effects on the in vitro opioid activity profile. While Guan-Dmt-Tic-Phe-Phe-NH₂ (**4**) retained very high δ and μ receptor binding affinities ($K_i = 0.146$ nM, $K_i^\mu = 0.518$ nM), it showed significant κ receptor binding affinity ($K_i = 35$ nM), in contrast to the low affinity of the non-guanidylated peptide ($K_i > 1$ μ M). Surprisingly, this compound turned out to be a potent full agonist in the MVD assay ($IC_{50} = 1.72$ nM). The effect was naloxone-reversible ($K_e = 0.308 \pm 0.57$ nM), indicating that it was mediated by opioid receptors. As expected on the basis of its high μ receptor binding affinity, this compound also showed high μ agonist potency in the GPI assay ($IC_{50} = 8.09$ nM). Guan-Dmt-Tic-Phe-Phe-NH₂ thus represents a potent, balanced μ agonist/ antagonist.

A study of flexible docking of compounds **4a** and **4** to the μ opioid receptor was performed using Mosberg's models of the receptor in the inactive and activated state.¹⁷ The mixed μ agonist/ antagonist **4a** and the mixed μ agonist/ antagonist **4** were docked to the inactive and the activated form of the receptor, respectively (Fig. 1). In general, a comparison of the ligand-receptor interactions of **4a** bound to the inactive receptor form with those of **4** bound to the activated form revealed that most of the interactions involved the same lipophilic receptor residues, including Tyr¹²⁹, Phe¹³³, Val²¹⁷, Phe²¹⁸, Ile²⁷⁷, Val²⁸¹, Leu²⁰⁰, Trp²⁸⁴, Leu²⁹⁹ and Met¹⁹⁹. Both the N-terminal amino group of **4a** and the N-terminal guanidino group of **4** were engaged in an electrostatic interaction (salt bridges) with Asp¹²⁸ in the third transmembrane helix of the receptor. However, due to the steric bulk of the guanidino group, peptide **4** was shifted relative to the position of peptide **4a** (average RMS deviation = 1.06 Å). This resulted in somewhat different interactions with corresponding receptor residues which in some cases also have different side chain orientations between the two receptor forms. These distinct receptor interactions may explain the antagonist vs. agonist behavior of compounds **4a** and **4**.

Compared to guanidylated peptide **4**, the pseudopeptide Guan-Dmt-Tic [CH₂NH]Phe-Phe-NH₂ (**5**) showed a similar opioid receptor binding profile with high μ and δ receptor binding affinities and moderate κ receptor binding affinity. In the GPI assay it was a 7-fold more potent μ opioid agonist ($IC_{50} = 1.22$ nM) than its nonguanidylated parent (**5a**) and, as was the case with **4**, it behaved as a full agonist in the MVD assay with subnanomolar potency ($IC_{50} = 0.750$ nM).

In comparison with peptide **4a**, its Lys³-analogue, H-Dmt-Tic-Lys-Phe-NH₂ (**6a**) showed similar subnanomolar receptor binding affinity, 16-fold lower μ receptor affinity and > 1000 -fold higher κ receptor affinity. In the functional assays **6a** displayed a mixed μ agonist/ antagonist profile similar to that of **4a**. The fact that despite the reduced μ receptor binding

affinity of compound **6a**, its agonist potency in the GPI assay is similar to that of **4a** can be explained with its high κ receptor binding affinity. In addition to μ receptors the GPI also contains κ receptors and their activation by **6a** compensated for the reduced μ receptor affinity of **6a** as compared to **4a**. Interestingly, the guanidinylated analogue of **6a**, Guan-Dmt-Tic-Lys-Phe-NH₂ (**6**) showed 7-fold lower κ receptor affinity and 55-fold higher μ receptor affinity than **6a** and comparable κ receptor affinity. The subnanomolar μ receptor affinity of **6** explains its extraordinary agonist potency (IC₅₀ = 0.289 nM) in the GPI assay. As was the case with guanidinylated peptides **4** and **5**, compound **6** was a full agonist in the MVD assay. Its somewhat lower agonist potency (IC₅₀ = 4.69 nM) is in agreement with its relatively lower κ receptor binding affinity (K_i = 2.20 nM).

The receptor binding profile of the pseudopeptide H-Dmt-Tic [CH₂ NH]Lys-Phe- NH₂ (**7a**) was similar to that of **6a**, except for its about 9-fold lower κ receptor affinity. Compound **7a** was a full agonist in the GPI assay with 3-fold higher potency than **6a**, but unlike **6a** it behaved as a partial agonist in the MVD assay. In comparison with **7a**, its guanidinylated derivative (**7**) showed a similar opioid receptor binding profile, comparable agonist activity in the GPI assay and similar partial agonist activity in the MVD assay.

Guanidinylation of the dipeptide antagonist H-Dmt-Tic-OH¹¹ (**8a**) had quite a drastic effect on the opioid activity profile. While Guan-Dmt-Tic-OH (**8**) had κ receptor binding affinity similar to that of **8a**, its μ receptor affinity was about 100-fold increased and it showed partial agonist activity in both the MVD assay and the GPI assay. Thus, Guan-Dmt-Tic-OH is a μ partial agonist/ κ partial agonist with low nanomolar binding affinity for μ and κ receptors and with no affinity for σ receptors at concentrations up to 10 μ M.

In conclusion, N-terminal amidination of various peptides of the TIPPP family had unexpected, diverse effects on the opioid activity profile. With one or two exceptions the guanidinylated derivatives showed similar or enhanced μ and κ receptor binding affinities as compared to their non-guanidinylated parent peptides. In the case of compounds **4** and **5** guanidinylation resulted in a significant enhancement of κ receptor binding affinity. Amidination also produced unexpected effects on the efficacy of the compounds, primarily at the κ receptor. The guanidinylated derivatives of the antagonists **1a**, **2a**, **3a** and **8a** turned out to be partial agonists with subnanomolar or low nanomolar κ receptor binding affinity. An interesting case is the dipeptide Guan- Dmt-Tic-OH (**8**) which retained high κ receptor affinity, showed greatly enhanced μ receptor binding affinity as compared to H-Dmt-Tic-OH (**8a**) and turned out to be a mixed μ partial agonist/ κ partial agonist. Also unexpected was the conversion of the mixed μ agonist/ κ antagonists **4a** and **5a** to mixed μ agonist/ full agonists (compounds **4** and **5**). In the case of peptides **4a** and **4** the results of a κ receptor docking study indicated a slightly shifted position of receptor-bound **4** relative to that of receptor-bound **4a** as a consequence of the increased steric bulk of the guanidino group as compared to the amino group in the electrostatic interaction with the Asp¹²⁸ residue of the receptor. This results in the stabilization of side chain orientations of lipophilic residues of the binding site seen in the activated form of the receptor and may explain the conversion from antagonism to agonism. Peptides **4** and **5** with a mixed μ agonist/ κ agonist profile are of interest because there is strong evidence to indicate that compounds with this profile may be effective for the treatment of pain with reduced side effects.¹⁸ The Lys³-analogues of DIPP-NH₂ and DIPP-NH₂[] (guanidinylated or non-guanidinylated) are essentially mixed μ / κ agonists with a κ antagonist, full agonist or partial agonist component. Compounds with mixed μ / κ opioid receptor agonist activity have therapeutic potential for treatment of cocaine abuse.¹⁹

Supplementary Material

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Acknowledgments

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Abbreviations

BBB	blood-brain barrier
Boc	<i>tert</i> -butoxycarbonyl
Cl-HOBt	6-chloro- 1-hydroxybenzotriazole
DAMGO	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol
DIC	1,3- diisopropylcarbodiimide
DIPP	H-Dmt-Tic-Phe-Phe-OH
DIPP-NH₂	H-Dmt-Tic-Phe- Phe-NH ₂
DIPP-NH₂[]	H-Dmt-Tic [CH ₂ NH]Phe-Phe-NH ₂
Dmt	2 ,6 -dimethyltyrosine
DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
ES-MS	electrospray mass spectrometry
GPI	guinea pig ileum
Guan	guanidino
HBUTU	2-(1H-benzotriazol-1-yl)- 1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	high performance liquid chromatography
MVD	mouse vas deferens
Tic	1,2,3,4-tetrahydroisoquinoline-3- carboxylic acid
TIPP	H-Tyr-Tic-Phe-Phe-OH
TIPP[]	H-Tyr-Tic [CH ₂ NH]Phe-Phe- OH
U69,593	(5 ,7 ,8 -(–)- <i>N</i> -methyl- <i>N</i> -[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide

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Figure 1.

Receptor docking studies. H-Dmt-Tic-Phe-Phe-NH₂ (**4a**) in green bound to the receptor in the inactive state (key residues depicted in white and Asp¹²⁸ in red), and Guan-Dmt-Tic-Phe-Phe-NH₂ (**4**) in magenta bound to the receptor in the activated state (key residues depicted in yellow and Asp¹²⁸ in orange).

Table 1

Receptor binding affinities of TIPP- and TIPP-NH₂ analogues^a

Compound	K _i [nM]	K _i ^p [nM]	K _i [nM]	Selectivity ratio (/μ /)
1 Guan-Tyr-Tic-Phe-Phe-OH	2.29 ± 0.51	875 ± 21	> 5000	1/382/> 2180
1a H-Tyr-Tic-Phe-Phe-OH ^b	1.22 ± 0.07	1720 ± 50	> 1000	1/1410/> 820
2 Guan-Dmt-Tic-Phe-Phe-OH	0.146 ± 0.007	126 ± 10	2260 ± 10	1/863/15500
2a H-Dmt-Tic-Phe-Phe-OH ^b	0.248 ± 0.025	141 ± 25	> 1000	1/569/> 4030
3 Guan-Tyr-Tic [CH ₂ NH]Phe-Phe-OH	0.968 ± 0.011	704 ± 82	> 10000	1/727/> 10300
3a H-Tyr-Tic [CH ₂ NH]Phe-Phe-OH ^b	0.308 ± 0.060	3230 ± 440	> 1000	1/10500/> 3250
4 Guan-Dmt-Tic-Phe-Phe-NH ₂	0.146 ± 0.041	0.518 ± 0.047	35.8 ± 3.0	1/4/245
4a H-Dmt-Tic-Phe-Phe-NH ₂ ^b	0.118 ± 0.016	1.19 ± 0.11	> 1000	1/10/> 8470
5 Guan-Dmt-Tic [CH ₂ NH]Phe-Phe-NH ₂	0.789 ± 0.141	1.02 ± 0.19	40.1 ± 10.1	1/1/51
5a H-Dmt-Tic [CH ₂ NH]Phe-Phe-NH ₂ ^b	0.447 ± 0.007	0.943 ± 0.052	> 1000	1/2/> 2240
6 Guan-Dmt-Tic-Lys-Phe-NH ₂	2.20 ± 0.38	0.354 ± 0.005	1.69 ± 0.09	1/0.2/0.8
6a H-Dmt-Tic-Lys-Phe-NH ₂	0.306 ± 0.061	19.4 ± 2.2	2.23 ± 0.17	1/63/7
7 Guan-Dmt-Tic [CH ₂ NH]Lys-Phe-NH ₂	1.83 ± 0.41	18.1 ± 3.1	24.7 ± 1.1	1/10/13
7a H-Dmt-Tic [CH ₂ NH]Lys-Phe-NH ₂	2.73 ± 0.80	9.11 ± 0.97	2.06 ± 0.59	1/3/0.8
8 Guan-Dmt-Tic-OH	2.66 ± 0.10	15.0 ± 1.3	> 10000	1/6/> 3760
8a H-Dmt-Tic-OH ^b	1.64 ± 0.07	1360 ± 160	> 1000	1/829/> 714

^aMean of 3 determinations ± SEM.

^bData taken from ref. ³

Table 2Opioid activities of TIPP- and TIPP-NH₂ analogues

Compound	MVD		GPI
	K _e [nM] ^a	IC ₅₀ [nM] ^a	IC ₅₀ [nM] ^a
1 Guan-Tyr-Tic-Phe-Phe-OH		P.A. (33%) ^c	inactive
1a H-Tyr-Tic-Phe-Phe-OH ^b	4.80 ± 0.20		inactive
2 Guan-Dmt-Tic-Phe-Phe-OH		1.57 ± 0.16 (IC ₃₅) ^d	1050 ± 150
2a H-Dmt-Tic-Phe-Phe-OH ^b	0.196 ± 0.008		inactive
3 Guan-Tyr-Tic [CH ₂ NH]-Phe-Phe-OH		P.A. (50%) ^c	inactive
3a H-Tyr-Tic [CH ₂ NH]-Phe-Phe-OH ^b	2.89 ± 0.14		inactive
4 Guan-Dmt-Tic-Phe-Phe-NH ₂		1.72 ± 0.19	8.09 ± 0.53
4a H-Dmt-Tic-Phe-Phe-NH ₂ ^b	0.209 ± 0.037		18.2 ± 1.8
5 Guan-Dmt-Tic [CH ₂ NH]Phe-Phe-NH ₂		0.750 ± 0.069	1.22 ± 0.18
5a H-Dmt-Tic [CH ₂ NH]Phe-Phe-NH ₂ ^b	0.537 ± 0.026		7.71 ± 0.31
6 Guan-Dmt-Tic-Lys-Phe-NH ₂		4.69 ± 0.94	0.289 ± 0.033
6a H-Dmt-Tic-Lys-Phe-NH ₂	2.18 ± 0.30		16.5 ± 1.9
7 Guan-Dmt-Tic [CH ₂ NH]Lys-Phe-NH ₂		7.35 ± 1.99 (IC ₃₅) ^d	9.46 ± 2.11
7a H-Dmt-Tic [CH ₂ NH]Lys-Phe-NH ₂		23.2 ± 2.66 (IC ₃₅) ^d	5.69 ± 0.90
8 Guan-Dmt-Tic-OH		P.A. (50%) ^c	P.A. (50%) ^c
8a H-Dmt-Tic-OH ^b	6.55 ± 0.27		Inactive

^aMean of 3 determinations ± SEM.^bData taken from ref.³^cPartial agonist (% of maximal inhibition of contractions).^dPartial agonist (IC₃₅).