Published in final edited form as:

Pept Sci (Hoboken). 2019 January ; 111(1): . doi:10.1002/pep2.24078.

Equipotent enantiomers of cyclic opioid peptides at μ opioid receptor

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Abstract

Head-to-tail cyclized analogues of the μ opioid receptor (MOR) agonist tetrapeptides DALDA (H-Tyr-D-Arg-Phe-Lys-NH₂) and [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂; Dmt = 2',6'-dimethyltyrosine) and their enantiomers (mirror-image isomers) were synthesized and pharmacologically characterized in vitro. Three pairs of enantiomeric cyclic peptides with both mirror-image isomers having equipotent MOR binding affinities but different binding affinities at the δ and κ opioid receptors were identified. The cyclic peptide enantiomers c[-D-Arg-Phe-Lys-Tyr-] (**1**) and c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**) showed nearly identical MOR binding affinity (1 – 2 nM) and equipotent MOR antagonist activity. The results of a MOR docking study indicated a very similar binding mode of the two enantiomers with nearly complete spatial overlap of the peptide ring structures and side chain interactions with the same MOR residues. Compounds **1** and **2** represent the first pair of enantiomeric G-protein-coupled receptor (GPCR) ligands having multiple chiral centers, with both optical antipodes showing equal, low nanomolar receptor binding affinity.

Graphical Abstract

1 | INTRODUCTION

Enantiomers (“mirror-image” isomers) of peptide hormones and neurotransmitters acting at G- protein-coupled receptors (GPCRs) are known to lack biological activity. This was demonstrated with all D-analogues of oxytocin,^[1] bradykinin,^[2,3] Val⁵-angiotensin II-Asp¹-β-amide^[4,5] and an eledoisin (5–11) 7-peptide analogue.^[6] In general, enantiomers of GPCR ligands with multiple chiral centers having equal binding affinity in the low nanomolar range have not been reported.

The opioid peptide analogue H-Tyr-D-Arg-Phe-Lys-NH₂ (DALDA) is a highly selective μ opioid receptor (MOR) agonist.^[7] Replacement of Tyr¹ in DALDA with 2',6'-dimethyltyrosine (Dmt) led to a compound, H-Dmt-D-Arg-Phe-Lys-NH₂ ([Dmt¹]DALDA), with subnanomolar MOR binding affinity and still high MOR binding selectivity.^[8] In the mouse- and rat tail-flick assays the antinociceptive potency of [Dmt¹]DALDA was 2–3 orders of magnitude higher than that of morphine with i.th. and s.c. administration.^[9–11] The Dmt residue also confers antioxidant activity to this compound and as a bifunctional MOR agonist/antioxidant [Dmt¹]DALDA was shown to be much more potent than morphine as an analgesic in two animal models of neuropathic pain.^[12,13] Furthermore, this compound has good drug-like properties, as indicated by its stability against enzymatic degradation, excellent blood-brain barrier (BBB) penetration and slow clearance.^[14]

Head-to-tail cyclization of [Dmt¹]DALDA resulted in a compound, c[-D-Arg-Phe-Lys-Dmt-] (“cyclodal”) (**5**), with very high subnanomolar MOR binding affinity ($K_i^\mu = 67$ pM) and MOR antagonist activity.^[15] The enantiomer of cyclodal, c[-Arg-D-Phe-D-Lys-D-Dmt-] (**6**), showed 20-fold lower, but still quite high MOR binding affinity ($K_i^\mu = 1.38$ nM). Here we describe a series of enantiomeric cyclic tetrapeptide pairs structurally related to the c[-D-Arg-Phe-Lys-Dmt-] (**5**)/c[-Arg-D-Phe-D-Lys-D-Dmt-] (**6**) pair in an effort to further explore structure-binding affinity and structure-function relationships of this type of mirror-image isomers.

c[-D-Arg-Phe-Lys-Tyr-] (**1**)

c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**)

c[-Arg-D-Phe-D-Lys-Tyr-] (**3**)
 c[-D-Arg-Phe-Lys-D-Tyr-] (**4**)
 c[-D-Arg-Phe-Lys-Dmt-] (**5**)^[15]
 c[-Arg-D-Phe-D-Lys-D-Dmt-] (**6**)^[15]
 c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-] (**7**)^[15]
 c[-Arg-D-Phe-D-LysΨ[CH₂NH]D-Dmt (**8**)

2 | MATERIALS AND METHODS

2.1 Chemistry

2.1.1 General—A Varian Prostar liquid chromatograph (Varian Scientific Instruments, Palo Alto, CA, USA) was used for the purification and purity assessment of the peptides. Preparative reversed-phase HPLC was performed on a Vydac 218-TP column (PROTO 300 C18 10 μm, 20 mm x 250 mm) with a linear gradient of 15 – 50 % MeOH in 0.1% trifluoroacetic acid (TFA) over 40 min at a flow rate of 12 mL/min, absorption being measured at 220 and 280 nm. Analytical reversed-phase HPLC was carried out on a Vydac 218-TP column (PROTO 300 C18 5 μm, 10 mm x 250 mm) with a linear gradient of 20 – 80% MeOH in 0.1 % TFA over 30 min at a flow rate of 1 mL/min. All compounds had >95% purity. The same column was used for the determination of the capacity factor *K'* under the same conditions. Precoated plates (silica gel 60 F₂₅₄, 250 μm, Merck, Darmstadt, Germany) were used for ascending thin layer chromatography (TLC) in the following systems: (I) *n*-BuOH/AcOH/H₂O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H₂O (15:10:3:2). Molecular masses of compounds were determined by electrospray mass spectrometry on a hybrid Q-ToF mass spectrometer interfaced to a Mass Lynx 4.0 data system. ¹H NMR spectra were recorded on a Varian INOVA 500 MHz spectrometer and referenced with respect to residual signals of the solvent. The following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Specific optical rotations were determined on an AA-5 automatic polarimeter.

2.1.2 Peptide synthesis

2.1.2.1 Synthesis of peptides 1 – 4: The compounds were prepared by cyclization between Lys and Phe or D-Lys and D-Phe of linear precursor peptides according to a synthetic scheme previously used for the preparation of cyclic peptide **5**.^[15] The linear precursor peptides were synthesized by the manual solid-phase technique using a 2-chlorotrityl resin (200 – 400 mesh, substitution 1.66 mequiv/g, Bachem Bioscience, King of Prussia, PA). 9-Fluorenylmethyloxycarbonyl (Fmoc) protection was used for the α-amino group of amino acids, and the side chains of Arg and Lys were protected with *tert*-butoxycarbonyl (Boc) and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc), respectively. *N,N*-dicyclohexylcarbodiimide (DIC)/6-chloro-*N*-hydroxybenzotriazole (6-Cl-HOBt) were used as coupling agents. Peptides were cleaved from the resin by treatment with 1% TFA in dichloromethane (DCM), followed by N-terminal Fmoc deprotection with 10% diethylamine (DEA) in DMF. After solvent evaporation the residue was triturated with anhydrous Et₂O,

yielding the peptides in solid form. The peptides were 90% pure as established by analytical HPLC and were used in the cyclization reaction without further purification.

For peptide cyclization, a solution of the side chain protected linear peptide precursor (0.1 mmol) and triethylamine (TEA) (11.4 μ L, 0.1 mmol) in 30 mL of anhydrous DMF was slowly added to a solution of benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (282 mg, 0.6 mmol) and 4-dimethylaminopyridine (DMAP) (102 mg, 0.8 mmol) in 300 mL of anhydrous DMF. The solution was stirred in the dark, and progress of the cyclization was monitored by analytical reversed-phase HPLC (linear gradient of 50 – 90 % MeOH in 0.1% TFA over 30 min at a flow rate of 1 mL/min). The time required for completion of the cyclization reaction varied from 2 to 24 h. After completion of the reaction the solvent was removed by vacuum distillation and Boc and Pmc side chain protection was removed by treatment with 95% TFA/H₂O for 4 h. After evaporation of the solvent and trituration of the residue with anhydrous Et₂O the peptides were obtained in solid form. Purification by preparative reversed-phase HPLC yielded the pure cyclic peptides. Yields for cyclization of the linear precursors were determined after side chain deprotection and final purification by reversed-phase HPLC.

***c[⁻D-Arg-Phe-Lys-Tyr-J* (1):** Cyclization yield 50%; HPLC K' 5.38; TLC R_f 0.43 (I), R_f 0.73 (II); HRMS (ESI) m/z calcd for C₃₀H₄₃N₈O₅ 595.3358, found 595.3356; [α]_D²⁰ -37.0° (c 0.5, 7% AcOH); ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.20 (s, 1H), 8.00 (d, J = 9.1 Hz, 1H), 7.87 (d, J = 9.0 Hz, 1H), 7.66 (d, J = 8.9 Hz, 2H), 7.48 (t, J = 5.2 Hz, 1H), 7.37 (d, J = 9.5 Hz, 1H), 7.32 – 7.26 (m, 3H), 7.25 – 7.19 (m, 4H), 6.96 (d, J = 8.3 Hz, 2H), 6.64 (d, J = 8.4 Hz, 2H), 4.46 (q, J = 8.8 Hz, 1H), 4.35 (dd, J = 16.6, 7.8 Hz, 1H), 4.26 (dd, J = 15.4, 8.6 Hz, 1H), 3.93 (q, J = 8.7 Hz, 1H), 3.04 – 2.88 (m, 7H), 2.76 – 2.68 (m, 4H), 1.71 – 1.04 (m, 12H).

***c[⁻Arg-D-Phe-D-Lys-D-Tyr-J* (2):** Cyclization yield 35%; HPLC K' 5.36; TLC R_f 0.43 (I), R_f 0.73 (II); HRMS (ESI) m/z calc. for C₃₀H₄₃N₈O₅ 595.3358, found 595.3349; [α]_D²⁰ +37.0° (c 0.5, 7% AcOH); ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.19 (s, 1H), 8.01 (d, J = 9.2 Hz, 1H), 7.87 (d, J = 9.1 Hz, 1H), 7.66 (d, J = 9.0 Hz, 2H), 7.48 (t, J = 5.6 Hz, 1H), 7.36 (d, J = 9.4 Hz, 1H), 7.32 – 7.27 (m, 3H), 7.23 – 7.19 (m, 4H), 6.96 (d, J = 8.5 Hz, 2H), 6.64 (d, J = 8.5 Hz, 2H), 4.46 (q, J = 8.6 Hz, 1H), 4.35 (dd, J = 16.6, 7.8 Hz, 1H), 4.26 (dd, J = 15.3, 9.0 Hz, 1H), 3.93 (q, J = 8.4 Hz, 1H), 3.04 – 2.88 (m, 7H), 2.75 – 2.68 (m, 4H), 1.74 – 1.04 (m, 12H).

***c[⁻Arg-D-Phe-D-Lys-Tyr-J* (3):** Cyclization yield 10%; HPLC K' 4.10; TLC R_f 0.33 (I), R_f 0.62 (II); HRMS (ESI) m/z calcd for C₃₀H₄₃N₈O₅ 595.3358, found 595.3354; [α]_D²⁰ +60.8° (c 0.5, 7% AcOH); ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.25 (s, 1H), 7.93 (dd, J = 16.0, 10.0 Hz, 2H), 7.68 (dd, J = 13.6, 8.2 Hz, 3H), 7.48 (m, 3H), 7.29 (t, J = 7.5 Hz, 3H), 7.25 – 7.11 (m, 6H), 6.98 (d, J = 8.5 Hz, 2H), 6.67 (d, J = 8.2 Hz, 2H), 4.47 (dd, J = 17.7, 10.1 Hz, 1H), 4.38 (dd, J = 17.6, 8.9 Hz, 1H), 3.98 (dt, J = 15.4, 7.5 Hz, 2H), 3.08 (dd, J = 13.2, 7.1 Hz, 3H), 1.66 – 1.09 (m, 10H).

***c[⁻D-Arg-Phe-Lys-D-Tyr-J* (4):** Cyclization yield 10%; HPLC K' 4.20; TLC R_f 0.34 (I), R_f 0.65 (II); HRMS (ESI) m/z calcd for C₃₀H₄₃N₈O₅ 595.3358, found 595.3354; [α]_D²⁰ -60.3°

(*c* 0.5, 7%, AcOH); ^1H NMR (500 MHz, DMSO-*d*₆) δ 9.25 (s, 1H), 7.93 (dd, *J* = 16.1, 10.1 Hz, 2H), 7.68 (dd, *J* = 13.8, 8.5 Hz, 3H), 7.48 (m, 3H), 7.29 (t, *J* = 7.5 Hz, 3H), 7.26 – 7.11 (m, 6H), 6.98 (d, *J* = 8.5 Hz, 2H), 6.67 (d, *J* = 8.2 Hz, 2H), 4.47 (dd, *J* = 17.7, 10.1 Hz, 1H), 4.38 (dd, *J* = 17.1, 9.6 Hz, 1H), 3.98 (td, *J* = 15.2, 7.7 Hz, 2H), 3.08 (dd, *J* = 13.2, 7.1 Hz, 3H), 1.66 – 1.05 (m, 10H).

2.1.2.2 Synthesis of c[-Arg-D-Phe-D-Lysψ[CH₂NH]D-Dmt-] (8): The compound was prepared according to a synthetic scheme previously used for the synthesis of cyclic peptide 7.^[15] The linear tripeptide H-D-Dmt-Arg(Tos)-D-Phe (Tos = *p*-toluenesulfonyl) was assembled by the manual solid-phase technique on a Sasrin resin (0.65 equiv/g, Bachem). To introduce the reduced peptide bond between the D-Lys and D-Dmt residues, a reductive alkylation reaction^[16] between Boc-D-Lys(Z-2-Cl)-aldehyde (Z-2-Cl = 2-chlorobenzylloxycarbonyl) and the α-amino group of the resin-bound tripeptide was performed. Boc-D-Lys(Z-2-Cl)-aldehyde was synthesized via preparation of Boc-D-Lys(Z-2-Cl)-*N*-methoxy-*N*-methylamide by using a published procedure.^[17] The peptide was then cleaved from the resin and N-terminally deprotected by treatment with a TFA/H₂O/TIS (9.5:0.25:0.25) (TIS = triisopropylsilane) solution for 3 h. After filtration and evaporation of the solvent the residue was triturated with Et₂O, yielding the crude, side chain protected tetrapeptide in solid form. The product was purified by preparative HPLC using a Vydac 218-TP column PROTO 300 C18 10 μm, 20 mm x 250 mm) with a linear gradient of 50 – 90% MeOH in 0.1% TFA over 40 min at a flow rate of 12 mL/min. Its structural identity was confirmed by mass spectrometry ([M+H⁺] = 949). Cyclization was performed as described above for the preparation of compounds 1 – 4. The cyclization was complete after 4 h, as determined by analytical reversed-phase HPLC. After evaporation of the DMF, the Z-2-Cl and Tos protection groups were removed by treatment with HF/anisole at 0 °C for 60 min. Following HF evaporation the residue was taken up in 7% AcOH, and after lyophilisation the target peptide was purified by preparative reversed-phase HPLC.

c[-D-Arg-Phe-Lysψ[CH₂NH]Dmt-] (7): Cyclization yield 35%; HPLC *K'* 5.38; TLC *R_f* 0.13(I), *R_f* 0.66(II); HRMS (ESI) *m/z* calcd for C₃₂H₄₉N₈O₄ 609.3877, found 609.3861; [*α*]_D²⁰ + 51.2° (*c* 0.5, 7% AcOH); ^1H NMR (500 MHz, DMSO-*d*₆) δ 9.07 (s, 1H), 7.78 (s, 3H), 7.47 (t, *J* = 5.5 Hz, 1H), 7.27-7.21 (m, 6H), 7.14 (d, *J* = 7.3 Hz, 1H), 6.41 (s, 2H), 4.26 (dd, *J* = 15.1, 7.8 Hz, 1H), 4.20 (dd, *J* = 15.5, 8.0 Hz, 1H), 3.01- 2.88 (m, 5H), 2.82-2.72 (m, 3H), 2.17 (s, 6H), 1.65 – 1.42 (m, 5H), 1.37-1.21 (m, 3H), 1.04 (m, 2H).

c[-Arg-D-Phe-D-Lysψ[CH₂NH]D-Dmt-] (8): Cyclization yield 40%; HPLC *K'* 5.38; TLC *R_f* 0.13(I), *R_f* 0.66(II); HRMS (ESI) *m/z* calcd for C₃₂H₄₉N₈O₄ 609.3877, found 609.3862; [*α*]_D²⁰ – 51.0° (*c* 0.5, 7% AcOH); ^1H NMR (500 MHz, DMSO-*d*₆) δ 9.04 (s, 1H), 7.74 (s, 3H), 7.45 (t, *J* = 5.5 Hz, 1H), 7.26 – 7.21 (m, 6H), 7.14 (d, *J* = 7.3 Hz, 1H), 6.49 (s, 2H), 4.26 (dd, *J* = 14.7, 8.4 Hz, 1H), 4.20 (dd, *J* = 15.5, 8.0 Hz, 1H), 2.94-2.89 (m, 5H), 2.78-2.72 (m, 3H), 2.20 (s, 6H), 1.65 – 1.42 (m, 5H), 1.36- 1.26 (m, 3H), 1.09-1.05 (m, 2H).

2.2 In vitro pharmacological evaluation

Opioid receptor binding studies were performed as described elsewhere.^[18] Binding affinities for μ and δ receptors were determined by displacing, respectively, tritiated H-Tyr-

D-Ala-Gly-N^αMePhe-Gly-ol ([³H]DAMGO) (Multiple Peptide Systems, San Diego, CA) and tritiated H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH ([³H]DSLET) (Multiple Peptide Systems) from rat brain membrane binding sites, and κ receptor binding affinities were measured by displacing tritiated (5α,7α,8β-(—)-*N*-methyl-*N*-[7-pyrrolidinyl-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide ([³H]U69,593) (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [³H]DAMGO, [³H]DSLET and [³H]U69,593 at respective concentrations of 0.72, 0.78 and 0.8 nM. IC₅₀ values were determined from log dose—response curves, and K_i values were calculated from the IC₅₀ values using the equation of Cheng and Prusoff,^[19] using values of 1.3, 2.6 and 2.3 nM for the dissociation constants of [³H]DAMGO, [³H]DSLET and [³H]U69,593, respectively.

The guinea pig ileum (GPI)^[20] and mouse vas deferens (MVD)^[21] functional assays were carried out as previously reported in detail.^[18,22] A dose—response curve was determined with [Leu⁵]enkephalin as standard for each ileum and vas preparation and IC₅₀ values of the compounds being tested were normalized according to a published procedure.^[23] K_e values for antagonists were determined from the ratios of IC₅₀ values obtained with an agonist in the presence and absence of a fixed antagonist concentration.^[24] μ antagonist K_e values were determined in the GPI assay against the μ agonist TAPP (H-Tyr-D-Ala-Phe-Phe-NH₂)^[7], with concentrations of antagonists **1**, **2**, **5** and **6** of 1000nM, 1000 nM, 20 nM and 200 nM, respectively. κ antagonist K_e values were also determined in the GPI assay against the κ agonist *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (U50,488), with concentrations of antagonists **1**, **2**, **5** and **6** of 2000 nM, >10000 nM, 500 nM and >1000 nM, respectively.

2.3 MOR docking studies

The generation of the lowest-energy conformation of enantiomers **1** and **2** was analogous to that described for c[-D-Arg-Phe-Lys-Dmt-] (**5**).^[15] All calculations were performed using SYBYL, version 7.0 (Tripos Associates, St. Louis, MO). Energy calculations were performed using the Tripos force field with a dielectric constant of 78. To determine low energy conformations of the cyclic peptide a stepwise approach was used.^[25] First, the bare ring structure was constructed, consisting of only the atoms directly attached to the ring, along with associated hydrogen atoms. After minimization, a systematic conformational grid search was performed to identify low energy structures, whereby each rotatable bond was rotated by 30° increments over all space. Structures without unfavorable vdw contacts permitting ring closure within 0.4 Å of a normal bond were identified and then minimized. The side chains in an extended conformation were then attached to the identified lowest-energy conformation of the bare peptide ring structure with all-*trans* peptide bonds and after reminimization the resulting structure was used for MOR docking.

Flexible docking to the MOR was performed using the software program GLIDE (Schrödinger LLC). The crystal structure of the MOR in the inactive form^[26] (RSCB PDB code 4DKL) was obtained from the RSCB database. A dielectric constant of 1 was used in the docking studies and in all subsequent calculations involving ligand—receptor complexes. Each of the resulting ligand—receptor complexes was minimized using the conjugate gradient approach.^[27] Molecular dynamics simulations of 100 ps at 300 K were

performed to assess the stability of each complex. In each case, no significant change in the complex structure was observed during the simulation.

3. | RESULTS AND DISCUSSION

3.1 In vitro opioid activity profiles of cyclic tetrapeptide enantiomers

The head-to-tail cyclized analogue of DALDA, c[-D-Arg-Phe-Lys-Tyr-] (**1**) (Figure 1) showed MOR binding affinity ($K_i^\mu = 1.04$ nM) (Table 1) comparable to that of linear DALDA ($K_i^\mu = 1.69$ nM).^[8] Its δ opioid receptor (DOR) and κ opioid receptor (KOR) binding affinities were more modest and, consequently, it exhibited considerable MOR binding selectivity (Table 1). In the GPI assay compound **1** displayed MOR antagonist activity against the MOR agonist TAPP ($K_e = 814$ nM) and KOR antagonist activity against the KOR agonist U50,488 ($K_e = 964$ nM) (Table 2). In the MVD assay (DOR-representative) it was a low efficacy partial agonist. Interestingly, the enantiomer of **1**, c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**) showed almost the same low nanomolar MOR binding affinity ($K_i^\mu = 2.30$ nM) as **1**. It had weak binding affinities for the DOR ($K_i^\delta = 2490$ nM) and KOR ($K_i^\kappa = 389$ nM) and, thus, was more MOR-selective than its enantiomer. In agreement with the MOR binding data, enantiomer **2** had the same MOR antagonist activity as enantiomer **1** ($K_e^\mu = 776$ nM versus $K_e^\mu = 814$ nM). Thus, the two enantiomers are equipotent MOR antagonists. Enantiomer **2** was characterized as a weak KOR antagonist ($K_e^\kappa = 11400$ nM) in the GPI assay and as a weak partial agonist in the MVD assay.

Replacement of D-Tyr in c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**) with Tyr resulted in an analogue, c[-Arg-D-Phe-D-Lys-Tyr-] (**3**), with about 75-fold lower MOR binding affinity ($K_i^\mu = 171$ nM). The compound showed no DOR binding affinity at 10 μ M and very weak KOR affinity. In the GPI assay it turned out to be a partial agonist (maximal inhibition of contractions = 60%) with an IC_{30} of 2610 nM. Its mirror-image isomer, c[-D-Arg-Phe-Lys-D-Tyr-] (**4**), had about the same MOR binding affinity ($K_i^\mu = 205$ nM) as its enantiomer, and no affinity for the DOR and KOR at concentrations up to 10 μ M. In the GPI assay it showed μ partial agonist activity with similar potency ($IC_{30} = 1430$ nM) and identical efficacy ($e = 0.6$) as its enantiomer. Thus, these two enantiomers are equipotent MOR partial agonists.

We previously reported^[15] that the head-to-tail cyclized analogue of [Dmt¹]DALDA, c[D-Arg-Phe-Lys-Dmt-] (**5**) ("cyclodal"), was a selective MOR antagonist with subnanomolar MOR binding affinity ($K_i^\mu = 63.7$ pM), high stability against enzymatic degradation and capability to penetrate the BBB. The 16-fold enhanced MOR binding affinity of c[-D-Arg-Phe-Lys-Dmt-] (**5**) as compared to c[-D-Arg-Phe-Lys-Tyr-] (**1**) can be explained with additional hydrophobic interactions of the two Dmt methyl groups with lipophilic receptor residues. The MOR binding selectivity of **5** was similar to that of **1**. **5** was characterized as a potent MOR antagonist ($K_e^\mu = 3.26$ nM) and a moderately potent KOR antagonist ($K_e^\kappa = 40.3$ nM). In comparison with **5**, its enantiomer, c[-Arg-D-Phe-D-Lys-D-Dmt-] (**6**), showed about 20-fold lower but still high MOR binding affinity ($K_i^\mu = 1.38$ nM) and higher MOR binding selectivity.^[15] In agreement with the receptor binding data, **6** had about 25-fold weaker μ antagonist activity ($K_e^\mu = 83.7$ nM) than **5** and weak κ antagonist activity $K_e^\kappa = 1320$ nM) in the GPI assay.

As previously reported^[15], reduction of the peptide bond between Lys and Dmt in c[-D-Arg-Phe-Lys-Dmt-] (**5**) resulted in a compound, c[D-Arg-Phe-LysΨ[CH₂NH]Dmt-] (**7**), with still quite high MOR binding affinity (K_i^{μ} = 9.02 nM) and modest binding affinities for the DOR and KOR. Interestingly, this compound turned out to be a full μ opioid agonist in the GPI assay and a weak partial agonist in the MVD assay. Thus, this compound is a selective MOR agonist. As a distinctive feature it contains a protonatable amino group at the reduced amide bond linking the Lys and Dmt residues. Its enantiomer, c[-Arg-D-Phe-D-LysΨ[CH₂NH]D-Dmt-] (**8**), had almost equal MOR binding affinity (K_i^{μ} = 13.8 nM) and weaker DOR and KOR binding affinities. It displayed MOR partial agonism in the GPI assay and was inactive in the MVD assay. Thus, these two enantiomers were about equipotent in terms of MOR binding affinity, but differed in their intrinsic efficacy at the MOR (full versus partial agonism).

The antagonist activities and agonist potencies determined with the cyclic tetrapeptides described here in the GPI assay were lower than expected in comparison with their receptor binding affinities. This discrepancy, generally observed with DALDA, [Dmt¹]DALDA and their analogues,^[8,15,28] likely is due to somewhat impeded access of this class of positively charged peptides to the receptors located in the ileum preparation. However, a good correlation between the potency ratios obtained in the GPI assay and those determined in the receptor binding assays is observed within the series of compounds tested.

3.2 | MOR docking studies

As described previously,^[15] we identified a lowest energy conformer of c[-D-Arg-Phe-Lys-Dmt-] (**5**) by NMR spectroscopy and theoretical conformational analysis, and performed a docking study with this structure and with the analogously determined structure of the mirror-image isomer c[-Arg-D-Phe-D-Lys-D-Dmt-] (**6**), using the crystal structure of the MOR in the inactive state.^[26] The results showed that the very high MOR binding affinity of c[-D-Arg-Phe-Lys-Dmt-] is due to a unique binding mode characterized by two salt bridges between the ligand's Lys side chain amino group and the MOR Asp127^{3.32} residue and between the ligand's D-Arg guanidine group and Glu229^{5.35} of the MOR, plus a hydrogen bond between the Dmt-OH group and His297^{6.52}. Despite its opposite stereochemistry the overall binding mode of the enantiomer, c[-Arg-D-Phe-D-Lys-D-Dmt-] was similar, except for a 2.2 Å shift in the positioning of the peptide backbone ring structure and the lack of a salt bridge between the D-Lys side chain and Asp147^{3.32} which may explain its 20-fold lower MOR binding affinity as compared to its enantiomer.

An analogous docking study was performed with c[-D-Arg-Phe-Lys-Tyr-] (**1**) and its equipotent enantiomer c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**). The results showed almost identical binding modes for the two enantiomers (Figure 2). There is an almost complete overlap of the two peptide backbone ring structures (rmsd = 0.24 Å). Salt bridges between the side chain amino group of Lys/D-Lys and Asp147^{3.32} and between the Arg/D-Arg guanidino group and Glu229^{5.35}, and an H-bond between the Tyr/D-Tyr hydroxyl group and His297^{6.52} are seen with both docked enantiomers. Furthermore, the comparison between docked c[D-Arg-Phe-Lys-Tyr-] and docked c[Arg-D-Phe-D-Lys-D-Tyr-] reveals similar side chain interactions with various MOR residues. In both cases Tyr/D-Tyr interacts with

Tyr148^{3.33}, Met151^{3.36}, Lys233^{5.39}, Val236^{5.42}, Trp293^{6.48}, Ile296^{6.51}, His297^{6.52} and Val300^{6.55}; Arg/D-Arg with Leu219 and Glu229^{5.35}; Lys/D-Lys with Asp147^{3.32}, Ile322^{7.39} and Tyr326^{7.43}; and Phe/D-Phe with Gln124^{2.60} and Asp127^{2.63}. In the case of the Phe residue a slight difference in the side chain orientation is seen with L-Phe $X_1 = 163^\circ$ and D-Phe $X_1 = 176^\circ$, which permits the two additional interactions of D-Phe in c[-Arg-D-Phe-D-Lys-D-Tyr-] with MOR residues Trp133^{2.69} and Val143^{3.28}. Because of the opposite stereochemistry the side chain orientations in the two enantiomers are somewhat different, but interactions with the same MOR residues are still possible because of side chain flexibility in the peptide ligand.

4. | CONCLUSIONS

Ligand stereoselectivity is generally observed with GPCRs. Opioid receptors are known to be highly stereoselective in binding classical opiates, with dextrorotatory isomers typically having 3 orders of magnitude lower MOR binding affinity than their levorotatory isomers. Examples are the enantiomeric pairs (-)-naloxone and (+)-naloxone, (-)-levorvanol and (+)-dextrorphan, and levo- and dextromorphinans.^[29,30]

The enantiomeric cyclic tetrapeptides described here have four chiral centers. In the case of the c[-D-Arg-Phe-Lys-Tyr-]/c[-Arg-D-Phe-D-Lys-D-Tyr-] pair both enantiomers have nearly equal low nanomolar binding affinity (1.04 and 2.30 nM, respectively). The performed docking study indicated that the peptide backbone ring structures of the two enantiomers are positioned in exactly the same location of the MOR binding site with almost complete spatial overlap. The side chain orientations in the two enantiomers are somewhat different as a consequence of the opposite stereochemistry. However, the conformational flexibility of the D-Arg/L-Arg and D-Lys/L-Lys side chains allows them to assume conformations in each isomer permitting salt bridge formation with Glu229^{5.35} and Asp147^{3.32} of the MOR, respectively. The phenol groups of Tyr/D-Tyr in the docked enantiomers show almost complete spatial overlap, permitting identical H-bond formation with His297^{6.52}. The Phe side chains in the two docked enantiomers have a slightly different orientation but engage in interactions with hydrophobic MOR residues in both cases. The almost identical receptor interactions of all four residues in the two enantiomers explain the nearly equal MOR binding affinities. Compounds **1** and **2** represent the first pair of enantiomeric GPCR ligands having multiple chiral centers, with both optical antipodes showing equal, low nanomolar receptor binding affinity.

The enantiomeric pairs c[D-Arg-Phe-Lys-D-Tyr-]/c[Arg-D-Phe-D-Lys-Tyr-] and c[-D-Arg-Phe-Lys-Ψ[CH₂NH]Dmt-]/c[-Arg-D-Phe-D-Lys-Ψ[CH₂NH]D-Dmt-] also show equipotent MOR binding affinity of the optical antipodes. Interestingly, while the two enantiomers of each of the three pairs (**1/2**, **3/4**, **7/8**) have equal MOR binding affinity, they differ in their DOR and KOR binding affinities.

It is interesting to compare the ability of the three pairs of enantiomers with equipotent MOR binding affinities to activate the MOR. The two enantiomers c[D-Arg-Phe-Lys-Tyr-] and c[-Arg-D-Phe-D-Lys-D-Tyr-] have nearly identical MOR antagonist activity (K_e of 814 nM and 776 nM, respectively), and the two enantiomers c[-D-Arg-Phe-Lys-D-Tyr-] and c[-

Arg-D-Phe-D-Lys-Tyr-] have nearly identical MOR partial agonist activity (same efficacy $e = 0.6$ and $IC_{30} = 1430$ nM and 2610 nM, respectively). On the other hand, c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-] is a full agonist, whereas its enantiomer c[-Arg-D-Phe-D-LysΨ[CH₂NH]D-Dmt-] is a partial agonist.

Acknowledgments

Funding information

National Institute on Drug Abuse, NIH, Grant Award Number DA004443

Canadian Institutes of Health Research, Grant Award Number MOP89716

This research was supported by grants from the National Institute on Drug Abuse, NIH (DA00443) and the Canadian Institutes of Health Research (MOP-89716).

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FIGURE 1. Chemical structures of c[-D-Arg-Phe-Lys-Tyr-] (**1**) (left panel) and c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**) (right panel).

FIGURE 2.

Docking of c[-D-Arg-Phe-Lys-Tyr-] (**1**) (magenta) and of its enantiomer c[-Arg-D-Phe-D-Lys-D-Tyr-] (**2**) (green) to the crystal structure of the MOR in the inactive state (RSCB PDB code 4DKL).^[26] MOR key residues: Asp147^{3.32}, Glu229^{5.85} (depicted in red); His297^{6.52} (depicted in blue).

TABLE 1

Opioid receptor binding affinities of peptide enantiomers^a

Compound	K _i ^μ [nM]	K _i ^δ [nM]	K _i ^κ [nM]	Selectivity ratio μ/δ/κ
1 c[-D-Arg-Phe-Lys-Tyr-]	1.04 ± 0.10	64.0 ± 7.1	67.4 ± 6.1	1/62/65
2 c[-Arg-D-Phe-D-Lys-D-Tyr-]	2.30 ± 0.03	2490 ± 90	389 ± 7	1/1080/169
3 c[-Arg-D-Phe-D-Lys-Tyr-]	171 ± 35	> 10,000	4850 ± 510	1/>58/28
4 c[-D-Arg-Phe-Lys-D-Tyr-]	205 ± 25	> 10,000	> 10,000	1/>50/>50
5 c[-D-Arg-Phe-Lys-Dmt-] ^b	0.0637 ± 0.0047	1.53 ± 0.08	5.11 ± 0.43	1/24/73
6 c[-Arg-D-Phe-D-Lys-D-Dmt-] ^b	1.38 ± 0.19	263 ± 6	405 ± 84	1/191/293
7 c[-D-Arg-Phe-Lys-Ψ(CH ₂ NH)]Dmt-] ^b	9.02 ± 1.51	422 ± 107	60.9 ± 0.6	1/47/7
8 c[-Arg-D-Phe-D-Lys-Ψ(CH ₂ NH)]D-Dmt-]	13.8 ± 1.5	4260 ± 440	280 ± 24	1/309/20

^aValues are mean ± SEM for n = 3.

^bData taken from ref. 15.

TABLE 2

Opioid antagonist and agonist activities of peptide enantiomers^a

Compound	GPI			MVD ^d	
	K _e ^μ [nM] ^b	K _e ^κ [nM] ^c	IC ₅₀ [nM]	IC ₅₀ [nM]	
1 c[-D-Arg-Phe-Lys-Tyr-]	814 ± 96	964 ± 4			PA
2 c[-Arg-D-Phe-D-Lys-D-Tyr-]	776 ± 73	11400 ± 700			PA
3 c[-Arg-D-Phe-D-Lys-Tyr-]			2610 ± 400 (IC ₃₀) ^e		PA
4 c[-D-Arg-Phe-Lys-D-Tyr-]			1430 ± 230 (IC ₃₀) ^e		PA
5 c[-D-Arg-Phe-Lys-Dmt-] ^f	3.26 ± 0.84	40.3 ± 2.6			PA
6 c[-Arg-D-Phe-D-Lys-D-Dmt-] ^f	83.7 ± 7.2	1320 ± 100			PA
7 c[-D-Arg-Phe-Lys-Ψ[CH ₂ NH]Dmt-] ^f			2510 ± 360		PA
8 c[-Arg-D-Phe-D-Lys-Ψ[CH ₂ NH]D-Dmt-]	PA ^d				inactive

^aValues are mean ± SEM for n = 3.

^bDetermined against TAPP.

^cDetermined against U50,488.

^dPA = partial agonist.

^ePartial agonist (maximal inhibition of contractions = 60%).

^fData taken from ref. 15.