Published in final edited form as:

J Med Chem. 2016 October 13; 59(19): 9243–9254. doi:10.1021/acs.jmedchem.6b01200.

A Cyclic Tetrapeptide (“Cyclodal”) and Its Mirror-Image Isomer Are Both High-Affinity μ Opioid Receptor Antagonists

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Abstract

Head-to-tail cyclization of the μ opioid receptor (MOR) agonist [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂ (**9**; Dmt = 2',6'-dimethyltyrosine) resulted in a highly active, selective MOR antagonist, c[-D-Arg-Phe-Lys-Dmt-] (**1**) (“cyclodal”), with subnanomolar binding affinity. A

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Supporting Information The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b01200.

1D and 2D NMR experiments establishing the all-*trans* peptide backbone conformation of cyclodal (**1**) (PDF)

3D coordinates of the MOR (inactive form) in complex with cyclodal (**1**) (Figure 3) (PDB)

3D coordinates of the MOR (inactive form) in complex with cyclodal (**1**) and c[-Arg-D-Phe-D-Lys-D-Dmt-] (**2**) (Figure 4) (PDB)

3D coordinates of the MOR (active form) in complex with c[-D-Arg-Phe-Lys-Ψ[CH₂NH]Dmt-] (**8**) (Figure 5) (PDB)

3D coordinates of all-*trans* low-energy conformer of cycodal (**1**) (Figure S-5) (PDB)

SMILES data (CSV)

The authors declare no competing financial interest.

DEDICATION Dedicated to the memory of Robert Schwyzler.

docking study of cyclodal using the crystal structure of MOR in the inactive form showed a unique binding mode with the two basic residues of the ligand forming salt bridges with the Asp¹²⁷ and Glu²²⁹ receptor residues. Cyclodal showed high plasma stability and was able to cross the blood–brain barrier to reverse morphine-induced, centrally mediated analgesia when given intravenously. Surprisingly, the mirror-image isomer (optical antipode) of cyclodal, c[-Arg-D-Phe-D-Lys-D-Dmt-] (**2**), also turned out to be a selective MOR antagonist with 1 nM binding affinity, and thus, these two compounds represent the first example of mirror image opioid receptor ligands with both optical antipodes having high binding affinity. Reduction of the Lys-Dmt peptide bond in cyclodal resulted in an analogue, c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-] (**8**), with MOR agonist activity.

INTRODUCTION

The dermorphin-derived tetrapeptide amide [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂; Dmt = 2',6'-dimethyltyrosine (**9**)) is a potent μ opioid receptor (MOR) agonist with subnanomolar MOR binding affinity and high MOR selectivity.¹ Its demonstrated resistance to enzymatic degradation, slow clearance, and ability to cross the blood–brain barrier are indicative of good druglike properties.^{2,3} When compared to morphine for analgesic activity in the mouse- and rat-tail-flick assays, [Dmt¹]DALDA was 3000-fold more potent with intrathecal (i.th.) administration⁴ and 400- to 220-fold more potent with subcutaneous (sc) administration^{5,6} and the duration of its antinociceptive effect was longer in this acute pain model.^{2,4} As a compound with combined μ opioid agonist and antioxidant activity, [Dmt¹]DALDA was also superior to morphine in producing antinociception in two animal models of neuropathic pain, the spinal nerve ligation model and the chronic post ischemia pain model of complex regional pain syndrome type I.^{7,8}

Cyclic opioid peptide analogues, including H-Tyr-c[N^γ-D-A₂bu-Gly-Phe-Leu-],⁹ H-Tyr-c[D-Cys-Gly-Phe-D-Cys]NH₂,¹⁰ H-Tyr-c[D-Pen-Gly-Phe-D-Pen]OH,¹¹ and H-Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13),¹² were first reported in the 1980s, and numerous cyclic opioid peptides of this type were subsequently synthesized. In these compounds the Tyr¹ residue is exocyclic with its protonable amino group capable of forming a salt bridge with the Asp residue in the third transmembrane helix (TMH) of opioid receptors, which is a requirement for opioid activity of most naturally occurring opioid peptides and their analogues. A naturally occurring cyclic tetrapeptide lacking a protonable amino group is the κ opioid receptor (KOR) selective antagonist c[-D-Pro-Phe-Trp-Phe-] (**11**) (CJ-15,208).¹³ SAR studies showed that the D-Trp analogue of **11** had increased KOR binding affinity^{14,15} and that alanine-substituted analogues of the latter peptide exhibited diverse opioid activity profiles.¹⁶ A head-to-tail cyclized endomorphin analogue c[-Tyr-D-Pro-D-Trp-Phe-Gly-] and the structurally related analogue c[-Tyr-Gly-D-Trp-Phe-Gly-] also lack a protonable nitrogen, and these cyclic pentapeptides showed MOR agonist or partial agonist activity.^{17,18}

Head-to-tail cyclized tetrapeptides are characterized by a very rigid 12-membered backbone ring structure. Because of their compacted structure and stability against enzymatic degradation, they are considered “druglike”. The unfavorable strain of the 12-membered ring is disadvantageous to their synthesis; however, incorporation of a D-amino acid, proline, or an N-alkylated amino acid facilitates cyclization. Here we report the successful synthesis

and pharmacological characterization of head-to-tail cyclized [Dmt¹]DALDA, c[-D-Arg-Phe-Lys-Dmt-], named “cyclodal” (**1**) (Figure 1). Cyclodal lacks a positively charged α -amino group at the Dmt residue but carries positive charges on the side chains of D-Arg and Lys and, therefore, must have a receptor binding mode distinct from that of the linear [Dmt¹]DALDA parent peptide.

Several decades ago the possibility that optical antipodes (mirror-image isomers) of peptide hormones and neurotransmitters acting at G-protein-coupled receptors may show some biological activity was examined. This turned out not to be the case, as all D-analogues of oxytocin,¹⁹ bradykinin,^{20,21} Val⁵-angiotensin II-Asp¹- β -amide,^{22,23} and an eledoisin (5–11) 7-peptide analogue²⁴ were inactive or exhibited extremely low activity. Since pairs of biologically active, head-to-tail cyclized tetrapeptides having mirror image relationship have not been previously reported, it was of interest to prepare and characterize the optical antipode of cyclodal, c[-Arg-D-Phe-D-Lys-D-Dmt-](**2**).

To examine the effect of the positive charge on the D-Arg and Lys side chains on the opioid activity profile, we also prepared cyclodal analogues in which D-Arg was replaced by D-citrulline (D-Cit) (**3**) and norleucine (Nle) was substituted for Lys (**4**). The effect of changing the length of the side chains of the basic amino acid residues was determined by substitution of D-homoarginine (D-hArg) for D-Arg (**5**) and of Orn for Lys (**6**). An analogue with an expanded (13-membered) ring structure was prepared by replacement of Lys with β -homolysine (β hLys) (**7**). To restore a positively charged α -amino group at the Dmt residue, a cyclodal analogue with a reduced peptide bond between the Lys and Dmt residues (c[-D-Arg-Phe-Lys Ψ [CH₂NH]Dmt-]) (**8**) was synthesized. This compound represents a novel type of head-to-tail cyclized pseudotetrapeptide.

RESULTS AND DISCUSSION

Chemical Synthesis

For the preparation of **1** (Scheme 1), cyclization between the Lys and Phe residues of the Lys-Dmt-D-Arg-Phe tetrapeptide sequence was performed. The linear precursor peptide was synthesized by the manual solid-phase peptide synthesis (SPPS) method using a 2-chlorotrityl resin, Fmoc protection of the α -amino group of amino acids, and DIC and 6-Cl-HOBt as coupling agents. Boc and Pmc protection was used for the side chains of Lys and D-Arg, respectively. After cleavage from the resin and Fmoc deprotection, cyclization was performed at high dilution using BOP and DMAP as coupling agents and was complete after a reaction time of 4 h. After removal of the Boc and Pmc side chain protecting groups the cyclic peptide was purified by preparative reversed-phase HPLC.

Most of the other cyclic tetrapeptides (**2–4**, **6**, **7**) were prepared in an analogous manner using corresponding linear precursor tetrapeptides and with cyclization being performed between the Lys (or corresponding residue) and the Phe (or D-Phe) residues. For the preparation of c[-D-hArg-Phe-Lys-Dmt-] (**5**) cyclization between the D-hArg and Dmt residues was performed (Scheme 2). The protected linear tetrapeptide N ^{α} -Boc-D-Lys(N ^{ϵ} -Fmoc)-Phe-Lys(Z-Cl)-Dmt was assembled on a 2-chlorotrityl resin. After Fmoc removal from the side chain of D-Lys, guanidinylation was performed using *N,N'*-bis-

(benzyloxycarbonyl)-1*H*-pyrazole-1-carboxamide. Following Boc deprotection and cleavage from the resin, cyclization was performed as described for the preparation of **1** and was complete after 4 h. In the synthesis of c[-D-Arg-Phe-Lys-Ψ[CH₂NH]Dmt-] (**8**) the reduced peptide bond was introduced in the linear precursor peptide by performing a reductive alkylation reaction between N^α-Boc-Lys(Z-2-Cl)-aldehyde and the resin-bound H-Dmt-D-Arg(Tos)-Phe tripeptide, and cyclization was performed between the Lys and Phe residues (Scheme 3).

Within the series of prepared cyclic tetrapeptides the time required for completion of the cyclization step varied from 4 to 48 h, and the yields of cyclization of the linear precursor peptides after deprotection and purification by reversed-phase HPLC ranged from 20% to 68%. Peptides were obtained in high purity (>95%). As expected for a pair of mirror-image isomers, the optical rotations of c[-D-Arg-Phe-Lys-Dmt-] (**1**) and c[-Arg-D-Phe-D-Lys-D-Dmt-] (**2**) are equal but of opposite sign (see “Experimental Section”).

In Vitro Biological Evaluation

Head-to-tail cyclization of [Dmt¹]DALDA resulted in a compound, c[-D-Arg-Phe-Lys-Dmt-] (“cyclodal”), with extraordinary MOR binding affinity in the subnanomolar range ($K_i^{\mu} = 63.7 \pm 4.7$ pM) and relatively lower DOR and KOR binding affinities, thus exhibiting marked MOR binding selectivity (Table 1). Interestingly, in the GPI assay it behaved as a potent MOR antagonist against the MOR agonist TAPP ($K_e = 3.26 \pm 0.84$ nM) (Table 2). It also antagonized the MOR agonist effects of its linear parent [Dmt¹]DALDA ($K_e = 3.12 \pm 0.20$ nM), morphine ($K_e = 3.06 \pm 0.62$ nM), and fentanyl ($K_e = 4.96 \pm 0.57$ nM). In that same assay it showed moderate KOR antagonist activity against the KOR agonists *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U50,488)²⁵ ($K_e = 40.3 \pm 2.6$ nM) and dynorphin A(1–13) ($K_e = 206 \pm 24$ nM). In the DOR-representative MVD assay it was characterized as a weak DOR partial agonist.

Surprisingly, the optical antipode of cyclodal, c[-Arg-D-Phe-D-Lys-D-Dmt-] (**2**) still retained high MOR binding affinity ($K_i^{\mu} = 1.38 \pm 0.19$ nM) and higher MOR vs DOR and MOR vs KOR binding selectivity as compared to cyclodal. In the GPI assay it showed MOR antagonist activity against TAPP ($K_e = 83.7 \pm 7.2$ nM) and morphine ($K_e = 51.4 \pm 12.6$ nM) and KOR antagonist activity against U50,488 ($K_e = 1320 \pm 100$ nM) and dynorphin A(1–13) ($K_e = 856 \pm 36$ nM). Like cyclodal, it exhibited weak DOR partial agonist activity in the MVD assay. In comparison with the widely used MOR antagonist CTOP,²⁶ this compound showed comparable MOR binding affinity, MOR antagonist activity and MOR vs DOR binding selectivity, and somewhat lower, but still high MOR vs KOR binding selectivity (Tables 1 and 2).

Deletion of the side chain positive charge of D-Arg by its replacement with D-citrulline (Cit) resulted in a compound, c[-D-Cit-Phe-Lys-Dmt-] (**3**) showing 130-fold reduced MOR binding affinity and a similar opioid receptor selectivity profile as the cyclodal parent. In agreement with the MOR binding affinity data, this analogue also showed 120-fold reduced MOR antagonist activity in the GPI assay. It behaved as a KOR antagonist in the latter assay and displayed weak DOR full agonist activity in the MVD assay. Substitution of norleucine (Nle) for Lys in cyclodal led to an analogue c[-D-Arg-Phe-Nle-Dmt-] (**4**) with 24-fold

reduced MOR binding affinity and with lower MOR binding selectivity. In the functional GPI assay it showed 5-fold lower MOR antagonist activity than cyclodal and similar KOR antagonist activity. The results obtained with these two compounds indicate that the positive charges on the D-Arg and Lys side chains of cyclodal are essential for its subnanomolar MOR binding affinity, presumably because of their ability to engage in salt bridge formation with negatively charged receptor moieties, as indicated by a MOR docking study (see below).

Elongation of the side chain of D-Arg by its replacement with D-homoarginine (D-hArg) resulted in a compound, c[D-hArg-Phe-Lys-Dmt-] (**5**), showing 2-fold higher MOR and DOR binding affinities and 7-fold higher KOR binding affinity. In comparison with the cyclodal parent, this analogue displayed a similar activity profile in the functional assays. An analogue with a shortened Lys side chain, c[-D-Arg-Phe-Orn-Dmt-] (**6**), showed an opioid receptor binding profile similar to that of its parent and slightly lower MOR and KOR antagonist activities in the GPI assay. The results obtained with compounds **5** and **6** indicate that variation of the side chain length of the D-Arg and Lys residues of cyclodal does not have a significant effect on the opioid activity profile. Expansion of the 12-membered peptide ring structure to a 13-membered one was achieved by replacement of the Lys residue with β -homolysine (β hLys). In comparison with the cyclodal parent, c[-D-Arg-Phe- β hLys-Dmt-] (**7**) displayed 2-fold lower MOR binding affinity, 11-fold lower DOR binding affinity, and 6-fold higher KOR binding affinity. In agreement with the receptor binding data, compound **7** was a 3-fold less potent MOR antagonist and a 2-fold more potent KOR antagonist as compared to cyclodal. These results indicate that the performed ring expansion did not have a drastic effect on the opioid activity profile.

Since a positively charged α -amino group of the N-terminal Tyr or Dmt residue of most opioid peptide agonists and antagonists is essential for high opioid receptor binding affinity, a cyclodal analogue with a reduced peptide bond between the Lys and Dmt residues was synthesized. The resulting pseudopeptide, c[-D-Arg-Phe-Lys Ψ [CH₂NH]Dmt-] (**8**), carries a positive charge on the secondary amine of the reduced peptide bond at physiological pH and is characterized by increased conformational flexibility of the peptide backbone at the -CH₂-NH- bond. This compound retained quite good MOR binding affinity ($K_i^H = 9.02$ nM) and still showed MOR binding selectivity. Most interestingly it turned out to be a full agonist in the GPI assay ($IC_{50} = 2510$ nM), about 10-fold weaker than [Leu⁵]enkephalin ($IC_{50} = 246$ nM).¹ To the best of our knowledge, this compound represents the first head-to-tail cyclized tetrapeptide containing a reduced peptide bond.

The cyclic tetrapeptides described here showed lower antagonist activities and agonist potency in the GPI assay than would be expected on the basis of their opioid receptor binding affinities. This discrepancy has been generally observed with [Dmt¹]DALDA and its analogues^{1,27} and can be explained with reduced access of this class of peptides to receptors in the ileum preparation. However, among the various compounds of the series there is quite good qualitative agreement between the potency ratios determined in the receptor binding assays and the K_e or IC_{50} values determined in the GPI assay. In the MVD assay, compounds **1**, **2**, **4**, **5**, **6**, **8**, and **10** showed low efficacy partial agonist activity which did not permit the determination of accurate IC_{50} values, and compound **7** was inactive.

Identification of Low-Energy Conformers and MOR Docking Studies

On the basis of 2D ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) and 2D total correlation spectroscopy (TOCSY) spectra, it was established that cyclodal assumed a conformation with all-*trans* peptide bonds in DMSO (see Supporting Information). In a theoretical conformational analysis (molecular mechanics study) the lowest-energy conformation of the bare peptide ring structure with all-*trans* peptide bonds was identified. The side chains in an extended conformation were then attached, and after reminimization the resulting structure was used for MOR docking. A flexible docking study with the lowest all-*trans* peptide bond conformer of cyclodal using the crystal structure of the MOR in the inactive state²⁸ was performed (Figure 3). The results indicated that docked cyclodal is located in the same area of the orthosteric binding site as the β -FNA μ antagonist in the MOR- β -FNA crystal structure. Upon docking, the peptide side chains underwent conformational changes. In Figure 2 the structure with the side chain conformations of receptor-bound cyclodal is shown. In the MOR-bound conformation of cyclodal the side chain of the Phe residue is in the *trans* configuration and the Dmt side chain is in the *g*⁻ configuration. The Lys side chain amino group forms a salt bridge with Asp¹²⁷ in the third TMH, and a second salt bridge is formed between the D-Arg guanidino group and Glu²²⁹. In addition, the Dmt-OH group engages in a hydrogen bond with His²⁹⁷. The crystal structures of the β FNA-bound MOR²⁸ and of the naltrindole-bound DOR²⁹ show the expected salt bridge between the nitrogen of the alkaloid and the Asp residue in the third TMH of the receptor, and in the crystal structure of the DOR bound to the peptide DIPP-NH₂ (H-Dmt-Tic-Phe-Phe-NH₂, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), a δ antagonist/ μ agonist, an expected salt bridge between the N-terminal amino group of this peptidic ligand and Asp¹²⁷ is also observed.³⁰ The unique binding mode of cyclodal with two salt bridges strongly contributing to the binding interaction energy may explain its very high, subnanomolar MOR binding affinity.

A theoretical conformational analysis (molecular mechanics study) performed with the optical antipode of cyclodal, c[-Arg-D-Phe-D-Lys-Dmt-], resulted in the expected mirror image lowest-energy conformer with all-*trans* peptide bonds. The results of a MOR (inactive form) docking study showed that the optical antipode bound in the same location of the orthosteric binding site as the cyclodal enantiomer with the side chain residues on the opposite side of the peptide ring structure making contact with receptor residues (Figure 4). The centroid of the peptide ring structure of the optical antipode was shifted by 2.2 Å relative to that of cyclodal. Nonetheless, the Arg guanidine group was again able to form a salt bridge with Glu²²⁹ and the Dmt hydroxyl group was again engaged in a hydrogen bond with His²⁹⁷. However, the Lys side chain amino group no longer formed a salt bridge with the Asp¹⁴⁷-COOH group; instead it was engaged in a hydrogen bond with the Asp¹⁴⁷ backbone CO group. The loss of the second salt bridge interaction may explain the somewhat lower MOR binding affinity of c[-Arg-D-Phe-D-Lys-Dmt-] compared to cyclodal. However, overall the results of the performed docking studies demonstrate why both cyclodal and its mirror image are able to bind to the MOR with high affinity. Similarities in side chain interactions with various other MOR residues between docked cyclodal and its docked optical antipode were observed. In both cases Dmt/D-Dmt interacted with Tyr¹⁴⁸, Met¹⁵¹, Lys²³³, Val²³⁶, Trp²⁹³, Ile²⁹⁶, His²⁹⁷, Val³⁰⁰, and Ile³²²; Phe/D-Phe with

Gln¹²⁴, Asn¹²⁷, Trp¹³³, and Val¹⁴³; Lys/D-Lys with Asp¹⁴⁷, Ile³²², and Tyr³²⁶; and Arg/D-Arg with Leu²¹⁹ and Glu²²⁹.

The results of the docking study suggest that cyclodal and its mirror image bind to overlapping MOR binding sites. The alternative of the two enantiomers binding to different binding pockets is less likely for the following reasons. First, multiple runs of docking cyclodal and its antipode were performed and nearly identical binding poses for each enantiomer were always observed. Second, in the binding displacement studies carried out with the two enantiomers biphasic binding isotherms were not observed, as would be the case if two distinct binding sites were involved.

In a theoretical conformational analysis the lowest-energy conformer of the MOR agonist pseudopeptide c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-], characterized by all-*trans* peptide bonds and by the *trans* configuration at the reduced peptide bond, was identified (Figure 2). A MOR docking study with this conformer was performed using the crystal structure of the MOR in its active form.³¹ In the receptor-bound conformation of c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-] the Phe side chain assumed the *g*⁻ configuration as opposed to the Phe *t* configuration in MOR (inactive form)-bound cyclodal, and the χ_1 angle of Dmt differed by about 60° from that seen in the cyclodal-MOR (inactive form) complex. The results indicated that again a salt bridge was formed between the Arg guanidine group and Glu²²⁹ (Figure 5). The Lys side chain was engaged in an H-bond with the Asp¹⁴⁷ backbone carbonyl and also formed an H-bond with the Asp¹⁴⁷ side chain COOH group. Furthermore, the NH of the reduced peptide bond formed a hydrogen bond with the Asp¹⁴⁷ backbone carbonyl. Unlike in the case of the MOR (inactive form)-bound antagonists cyclodal and c[-Arg-D-Phe-D-Lys-D-Dmt-], the Dmt hydroxyl group of the agonist c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-] docked to the MOR in the active form was not engaged in hydrogen bond formation. Further comparison of the cyclodal-MOR (inactive form) and c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-]-MOR (active form) complexes revealed a number of common ligand side chain–receptor residue interactions for Dmt (Met¹⁵¹, Lys²³³, Ile²⁹⁶, His²⁹⁷, Val³⁰⁰) and Phe (Gln¹²⁴, Asn¹²⁷, Trp¹³³, Val¹⁴³, Ile¹⁴⁴). However, differences were also seen. In contrast to the cyclodal-MOR (inactive form) complex, in the c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-]-MOR (active form) complex Dmt did not interact with Tyr¹⁴⁸, Trp²⁹³, Val²³⁶, and Ile³²² but with Phe²³⁷, and Phe did not interact with Cys²¹⁷ and Thr²¹⁸ but with His⁵⁴, Ser⁵⁵, and Asp¹⁴⁷.

Plasma Stability, Brain Uptake, and Antagonist Activity in Vivo

The plasma stability of [Dmt¹]DALDA has previously been established.² Like [Dmt¹]DALDA, cyclodal incubated in rat plasma at 37 °C was stable for up to 48 h (Figure 6). Under the same conditions [Leu⁵]enkephalin was rapidly degraded, with less than 15% remaining after 15 min of incubation, and with CTOP 25% degradation was observed after 2 h of incubation. For both [Dmt¹]DALDA and cyclodal it was determined that BSA absorption did not occur and, thus, did not contribute to plasma stability (see Supporting Information).

A study of brain uptake of cyclodal in comparison with its linear parent [Dmt¹]DALDA was performed. Peptides were administered to mice intravenously (iv) at a dose of 2 mg/kg and

were quantitated by LC–MS analysis. Cyclodal was detected in brain with the maximum brain concentration (0.6 ng/g) seen 30 min after administration (Figure 7). At that time point the brain concentration of [Dmt¹]DALDA was about 3-fold higher than that of cyclodal. The time course of brain concentration indicates that brain efflux of the cyclic peptide is faster as compared to the linear one. The brain/plasma AUC ratio of 1.72 determined for cyclodal at the 2 mg/kg dose indicates significant brain uptake and is relatively high because of the compound's rapid clearance from plasma. Despite the higher brain concentration of [Dmt¹]DALDA, its brain/plasma AUC ratio (0.07) at the 2 mg/kg dose is relatively lower due to its higher concentration in and slower clearance from plasma. Overall, the results indicate that cyclodal crosses the BBB to some extent but not quite as well as [Dmt¹]DALDA. The ability of [Dmt¹]DALDA (net charge 3+) to translocate across cells has been shown to be important for its distribution across the BBB.³² Cyclodal (net charge 2+) has the same amino acid sequence as [Dmt¹]DALDA, but the severe conformational restriction introduced through cyclization and the loss of a positively charged amino group at the Dmt residue may make translocation across cells somewhat less efficient. Nonetheless, cyclodal shows significant BBB penetration, unlike CTOP which does not enter the brain.³³

The ability of systemically administered cyclodal to antagonize morphine-induced antinociception was examined in the rat-tail-flick and hot plate tests. In the tail-flick assay cyclodal given iv at a dose of 5 mg/kg reduced the tail withdrawal latency (TWL) at 30 and 60 min after morphine 4 mg/kg sc injection (Figure 8) but not at a dose of 1 mg/kg. The heat withdrawal thresholds determined in the hot plate test indicated that morphine (4 mg/kg, sc) produced analgesia compared to vehicle at the 30 and 60 min time points, and morphine analgesia at the 30 min time point was reduced by cyclodal at a dose of 5 mg/kg (Figure 8) but not at the 1 mg/kg dose. These results indicate that cyclodal is capable of crossing the BBB to reduce morphine-induced antinociception dose dependently. However, in agreement with the results of the brain uptake study, its antagonistic effect was of relatively short duration as a consequence of quite rapid brain efflux.

CONCLUSIONS

Head-to-tail cyclization of [Dmt¹]DALDA produced a highly active, selective MOR antagonist, “cyclodal”, with subnanomolar binding affinity. In comparison with the widely used MOR antagonist CTOP,²⁶ cyclodal showed 20-fold higher MOR binding affinity and 13-fold higher MOR antagonist activity in the functional assay. Docking cyclodal in its lowest-energy all-*trans* conformation to the MOR in its inactive form revealed that its extraordinary binding affinity is due to a unique binding mode characterized by the formation of two salt bridges between the side chains of its basic residues and Asp/Glu receptor residues. As expected for a cyclic tetrapeptide of this type, cyclodal showed druglike properties due to its rigid 12-membered peptide ring structure. It was found to be totally stable in plasma and capable of reversing morphine-induced, centrally mediated analgesia. However, in comparison with the linear, conformationally more flexible parent peptide [Dmt¹]DALDA, it showed somewhat lower brain uptake, indicating that structural flexibility of the peptide backbone favors effective BBB crossing of these tetrapeptides with the structural motif of alternating aromatic and basic amino acid residues.

Compared to cyclodal, its mirror image, c[-Arg-D-Phe-D-Lys-D-Dmt-], showed somewhat lower but still high MOR binding affinity and higher MOR binding selectivity. Opposite faces of the peptide ring structure of the two optical antipodes are engaged in the interaction with the MOR orthosteric binding site as a result of the opposite stereochemistry. The results of the docking studies showed that despite the somewhat different side chain orientations, the overall binding mode of c[Arg-D-Phe-D-Lys-D-Dmt-] is similar to that of cyclodal. This is made possible by a shift in the positioning of the peptide ring structure and by the conformational flexibility of the amino acid side chains. However, the salt bridge between the D-Lys side chain and the Asp¹⁴⁷ side chain carboxylate is no longer formed, and this may explain the somewhat lower MOR binding affinity as compared to cyclodal.

Compounds **1** and **2** represent the first example of mirror-image opioid receptor ligands with both optical antipodes having high MOR binding affinity. In the case of classical opiates with only one chiral center, the dextrorotatory isomers typically have 3 order-of-magnitude lower binding affinity at opioid receptors than their levorotatory isomers. Examples are the enantiomeric pairs (-)-naloxone and (+)-naloxone, (-)-levorphanol and (+)-dextrorphan, and levo- and dextro-morphinans.^{34,35} Cyclodal and its optical antipode have four chiral centers and the two enantiomers both have a structurally rigid peptide backbone but distinct side chain topologies. The results of the performed MOR docking study indicate that side chain structural flexibility and slightly different positioning of the peptide ring structure at the MOR binding site permit both enantiomers to adopt similar binding modes, resulting in high binding affinity for both of them. Enantiomeric peptide ligands of GPCRs with both isomers having high binding affinity are scarce. A 21-peptide and its all-D analogue were reported to have respective binding affinities of 456 nM and 32 nM for the CXCR4.³⁶

The results of SAR studies performed with cyclodal indicated that the positive charges on the D-Arg and Lys side chains are important for high MOR binding affinity. Variation of the side chain length of the two basic residues or expansion of the 12-membered peptide ring structure to a 13-membered one did not greatly affect the opioid activity profile.

The pseudopeptide analogue c[-D-Arg-Phe-LysΨ[CH₂NH]Dmt-] represents the first head-to-tail cyclized tetrapeptide containing a reduced peptide bond and, interestingly, showed MOR agonist activity. Its lowest-energy, all-*trans* peptide backbone conformation is similar to that of cyclodal. However, when docked to the crystal structure of the MOR in its active form, some differences in side chain orientations and receptor interactions are seen as compared to the cyclodal antagonist docked to the crystal structure of the MOR in the inactive form.

EXPERIMENTAL SECTION

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 (22 mm × 250 mm) with a linear gradient of 15–50% MeOH in 0.1% 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 (10 mm × 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 factors K' under the same conditions. Precoated plates (silica gel 60 F₂₅₄, 250 μ m, Merck, Darmstadt, Germany) were used for ascending TLC in the following solvent 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-T of 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 in reporting spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Specific optical rotations were determined on an AA-5 automatic polarimeter.

Peptide Synthesis. Synthesis of 1, 2, 3, and 4

The compounds were prepared by cyclization between Lys (or corresponding residue) and Phe (or D-Phe) of linear precursor peptides. 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). Fmoc protection was used for the α -amino group of amino acids, and the side chains of Arg and Lys were protected with Boc and Pmc, respectively. DIC/6-Cl-HOBt were used as coupling agents. Peptides were cleaved from the resin by treatment with 1% TFA in DCM, followed by N-terminal Fmoc deprotection with 10% DEA in DMF. After solvent evaporation the residue was triturated with anhydrous Et₂O, yielding the crude peptides in the form of tan-colored solids. 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 TEA (11.4 μ L, 0.1 mmol) in 30 mL of anhydrous DMF was slowly added to a solution of BOP (282 mg, 0.6 mmol) and 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 4 to 48 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. A homogeneous cyclic monomer was obtained in all cases, and cyclodimerization was not observed. 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-Dmt-] (Cyclodal) (1)—Cyclization yield 68%; HPLC K' 5.58; TLC R_f 0.25 (I), R_f 0.71 (II); HRMS (ESI) m/z calcd for C₃₂H₄₆N₈O₅ 623.3669, found 623.3655; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.94 (s, 1H), 8.19 (d, J = 8.9 Hz, 1H), 8.04 (d, J = 8.8 Hz, 1H), 7.53 (s, br, 3H), 7.36 (d, J = 9.3 Hz, 2H), 7.32–7.19 (m, 7H), 6.37 (s, 2H), 4.67–4.44 (m, 2H), 4.30 (dd, J = 16.4, 8.8 Hz, 1H), 3.86 (dd, J = 17.0, 8.1 Hz, 1H), 3.10–

2.85 (m, 7H), 2.80 (dd, $J = 14.1, 6.1$ Hz, 2H), 2.70 (s, br, 3H), 2.22 (s, 6H), 1.67–1.29 (m, 9H), 1.16–1.05 (m, 1H), 0.97–0.86 (m, 1H); $\alpha_D^{20} -40.5^\circ$ (c 0.5, 7% AcOH).

c[α -Arg-D-Phe-D-Lys-D-Dmt-] (2)—Cyclization yield 40%; HPLC K' 5.58; TLC R_f 0.27 (I), R_f 0.66 (II); HRMS (ESI) m/z calcd for $C_{32}H_{46}N_8O_5$ 623.3669, found 623.3670; ^1H NMR (500 MHz, DMSO- d_6) δ 8.92 (s, 1H), 8.22 (d, $J = 8.9$ Hz, 1H), 8.01 (d, $J = 8.8$ Hz, 1H), 7.55 (s, br, 3H), 7.36 (d, $J = 9.3$ Hz, 2H), 7.35–7.20 (m, 7H), 6.37 (s, 2H), 4.67–4.40 (m, 2H), 4.30 (dd, $J = 16.4, 8.8$ Hz, 1H), 3.86 (dd, $J = 17.0, 8.1$ Hz, 1H), 3.11–2.85 (m, 7H), 2.82 (dd, $J = 14.1, 6.1$ Hz, 2H), 2.70 (s, br, 3H), 2.22 (s, 6H), 1.67–1.29 (m, 9H), 1.16–1.05 (m, 1H), 0.97–0.86 (m, 1H). $\alpha_D^{20} +40.4^\circ$ (c 0.5, 7% AcOH).

c[α -D-Cit-Phe-Lys-Dmt-] (3)—Cyclization yield 32%; HPLC K' 5.76; TLC R_f 0.40 (I), R_f 0.78 (II); HRMS (ESI) m/z calcd for $C_{32}H_{45}N_7O_6$ 624.3510, found 623.3502; ^1H NMR (500 MHz, DMSO- d_6) δ 7.85 (d, $J = 9.5$ Hz, 1H), 7.77 (d, $J = 9.8$ Hz, 1H), 7.59 (d, $J = 8.2$ Hz, 1H), 7.48 (d, $J = 8.5$ Hz, 1H), 7.29–7.11 (m, 6H), 6.34 (s, 2H), 5.89 (t, $J = 12.5$ Hz, 1H), 5.37 (s, 2H), 4.42–4.34 (m, 1H), 4.19 (dd, $J = 14.5, 8.0$ Hz, 1H), 4.16–4.10 (m, 1H), 4.04 (dd, $J = 17.9, 8.7$ Hz, 1H), 3.00 (dd, $J = 13.5, 8.6$ Hz, 1H), 2.90 (dd, $J = 13.5, 7.4$ Hz, 4H), 2.78–2.56 (m, 5H), 2.24–2.17 (m, 7H), 1.89 (s, 1H), 1.63–1.34 (m, 3H), 1.24 (t, $J = 17.6$ Hz, 1H), 0.99 (s, 1H), 0.79 (s, 1H).

c[α -D-Arg-Phe-Nle-Dmt-] (4)—Cyclization yield 20%; HPLC K' 6.86; TLC R_f 0.66 (I), R_f 0.83 (II); HRMS (ESI) m/z calcd for $C_{32}H_{45}N_7O_5$ 608.3560, found 608.3561; ^1H NMR (500 MHz, DMSO- d_6) δ 8.90 (s, 1H), 8.32 (d, $J = 9.2$ Hz, 1H), 7.93 (d, $J = 8.8$ Hz, 1H), 7.47 (d, $J = 9.7$ Hz, 2H), 7.31–7.26 (m, 3H), 7.20 (dd, $J = 17.7, 10.5$ Hz, 6H), 4.48 (dd, $J = 11.4, 6.4$ Hz, 2H), 4.30 (dd, $J = 15.9, 8.5$ Hz, 1H), 3.81 (q, $J = 8.6$ Hz, 1H), 3.29 (s, 1H), 3.10–2.97 (m, 4H), 2.96–2.84 (m, 4H), 2.80 (dd, $J = 14.2, 6.1$ Hz, 2H), 2.22 (s, 7H), 2.03–1.88 (m, 2H), 1.67–1.52 (m, 4H), 1.51–1.29 (m, 5H), 1.30–1.16 (m, 8H), 1.05 (dt, $J = 14.3, 7.0$ Hz, 2H), 0.97–0.88 (m, 1H), 0.83 (t, $J = 7.3$ Hz, 3H).

Synthesis of 5

The linear tripeptide H-Phe-Lys(Z-2-Cl)-Dmt was assembled by solid phase synthesis using Fmoc-protected amino acids, as described above for the syntheses of the linear precursors of compounds **1–4**. N^{α} -Boc-D-Lys(N^{ϵ} -Fmoc)-OH was then coupled to the resin-bound tripeptide, and after Fmoc removal guanidinylation of the Lys side chain amino group was performed using 2 equiv of N,N -bis(benzyloxycarbonyl)-1*H*-pyrazole-1-carboxamide in THF/DMF (3:1) at room temperature overnight. Completion of the guanidinylation reaction was confirmed by the Kaiser ninhydrin test, and the peptide was cleaved from the resin and N-terminally deprotected by treatment with a 50% TFA/DCM solution (45 min). After evaporation of the solvent and trituration of the residue with cold, dry EtOH the peptide was obtained in solid form in 70% yield. The purity of the product (>95%) was established by analytical reversed-phase HPLC and its structural identity was confirmed by mass spectrometry ($[M + H]^+$ 1091, $[M + Na]^+$ 1113). Cyclization was performed as described above for the preparation of cyclodal (**1**) and its analogues. The cyclization reaction was complete after 4 h, as determined by analytical reversed-phase HPLC. After evaporation of the DMF the Z and Z-2-Cl side chain protecting groups were removed by treatment with

HF/anisole for 1 h at 0 °C. Following HF evaporation, the residue was washed with Et₂OH and dissolved in 7% AcOH. After lyophilization the peptide was purified by preparative reversed-phase HPLC.

c[*D*-hArg-Phe-Lys-Dmt-] (5)—Cyclization yield 40%; HPLC *K'* 5.50; TLC *R_f* 0.30 (I), *R_f* 0.74 (II); HRMS (ESI) *m/z* calcd for C₃₃H₄₈N₈O₅ 637.3826, found 637.3812; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.28 (d, *J* = 9.2 Hz, 1H), 8.00 (s, 1H), 7.49 (d, *J* = 9.4 Hz, 1H), 7.44 (t, *J* = 7.0 Hz, 1H), 7.32–7.20 (m, 7H), 6.36 (s, 2H), 4.54–4.43 (m, 2H), 4.28 (q, *J* = 7.2 Hz, 1H), 3.83 (q, *J* = 8.7 Hz, 1H), 3.09–2.77 (m, 7H), 2.72–2.61 (m, 3H), 2.56–2.53 (m, 1H), 2.48–2.45 (m, 1H), 2.38–2.35 (m, 1H), 2.22 (s, 6H), 1.90 (s, 2H), 1.67–1.54 (m, 3H), 1.50–1.34 (m, 5H), 1.24 (s, 1H), 1.21–1.04 (m, 3H), 0.97.

Syntheses of 6 and 7

The protocol for synthesizing these two compounds was the same as the one used for the preparation of compounds 1–4.

c[*D*-Arg-Phe-Orn-Dmt-] (6)—Cyclization yield 37%; HPLC *K'* 5.51; TLC *R_f* 0.39, *R_f* 0.74 (II); HRMS (ESI) *m/z* calcd for C₃₁H₄₄N₈O₅ 609.3513, found 609.3517; ¹H NMR (500 Mz, DMSO-*d*₆) δ 8.24 (s, 1H), 8.17 (d, *J* = 9.2 Hz, 1H), 7.70 (m, 2H), 7.56 (s, 1H), 7.32–7.19 (m, 7H), 6.36 (s, 2H), 4.45 (q, *J* = 8.5 Hz, 2H), 4.25 (q, *J* = 8.1 Hz, 1H), 3.89 (q, *J* = 8.3 Hz, 1H), 3.07–3.00 (m, 3H), 3.00–2.78 (m, 5H), 2.71–2.62 (m, 3H), 2.54 (s, 1H), 2.46 (s, 1H), 2.36 (s, 1H), 2.22 (s, 6H), 1.88 (s, 3H), 1.67 (d, *J* = 7.4 Hz, 2H), 1.58 (s, 2H), 1.34 (m, 4H), 1.24 (m, 2H).

c[*D*-Arg-Phe-β-hLys-Dmt-] (7)—Cyclization yield 66%; HPLC *K'* 5.87; TLC *R_f* 0.36 (I), *R_f* 0.71 (II); HRMS (ESI) *m/z* calcd for C₃₃H₄₄N₈O₅ 637.3826, found 637.3820; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.93 (s, 1H), 8.41 (d, *J* = 8.6 Hz, 1H), 7.80 (d, *J* = 9.2 Hz, 1H), 7.65 (s, 3H), 7.49–7.38 (m, 3H), 7.34 (d, *J* = 10.1 Hz, 2H), 7.32–7.16 (m, 7H), 6.36 (s, 2H), 4.49 (q, *J* = 7.4 Hz, 1H), 4.41 (q, *J* = 8.6 Hz, 1H), 4.22 (q, *J* = 7.4 Hz, 1H), 3.71 (s, 1H), 3.06 (s, 1H), 3.01–2.81 (m, 6H), 2.75–2.62 (m, 4H), 2.48–2.37 (m, 1H), 2.20 (s, 6H), 1.42 (s, 4H), 1.24 (s, 2H), 0.87 (m, 1H).

Synthesis of 8

The linear tripeptide H-Dmt-*D*-Arg(Tos)-Phe was assembled by solid phase synthesis using Fmoc-protected amino acids as described above for the syntheses of the linear precursors of compounds 1–4. To introduce the reduced peptide bond between the Lys and Dmt residues, a reductive alkylation reaction³⁷ between *N*^α-Boc-Lys(*N*^ε-Z-2-Cl)-aldehyde and the α-amino group of the resin-bound tripeptide was performed. Boc-Lys(Z-2-Cl)-aldehyde was synthesized via preparation of Boc-Lys(Z-2-Cl)-*N*-methoxy-*N*-methylamide by using a published procedure.³⁸ The peptide was then cleaved from the resin and N-terminally deprotected by treatment with 95% TFA/H₂O for 3 h. After filtration and evaporation of the TFA/H₂O the residue was triturated with Et₂O, yielding the crude, side chain protected tetrapeptide in solid form. The product was purified by preparative reversed-phase HPLC, and its structural identity was confirmed by mass spectrometry ([M + H]⁺ 949). Cyclization was performed as described above for the preparation of cyclodal (1) and its analogues. The

cyclization reaction was complete after 4 h as determined by analytical reversed-phase HPLC. After evaporation of the DMF, the Z-2-Cl and Tos protecting 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 lyophilization the target peptide was purified by preparative reversed-phase HPLC.

c[^D-Arg-Phe-LysΨ[CH₂NH]Dmt-] (8)—Cyclization yield 35%; HPLC *K'* 5.38; TLC *R_f* 0.13 (I), *R_f* 0.66 (II); HRMS (ESI) *m/z* calcd for C₃₂H₄₈N₈O₄ 609.3877, found 609.3861; 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 (d, *J* = 6.2 Hz, 4H), 7.21 (dt, *J* = 13.1, 4.4 Hz, 2H), 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, 5H), 1.65–1.41 (m, 5H), 1.37–1.21 (m, 3H), 1.04 (s, 2H).

Identification of Low-Energy Conformers and MOR Docking Studies

All calculations were performed using SYBYL, version 7.0 (Tripos Associates, St. Louis, MO). The Tripos force field was used for energy calculations, and the dielectric constant used was 78. A stepwise approach was used to determine low energy conformations of the cyclic peptides.³⁹ 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 ring structures. Each rotatable bond was rotated in 30° increments over all space. An allowed conformation was obtained if in a structure without unfavorable vdw contacts the ring could close within 0.4 Å of a normal bond. Each allowed ring structure was minimized. For the lowest energy ring structure, the side chains were attached and the structures were reminimized.

Flexible docking to the MOR was performed using the software program GLIDE (Schrödinger LLC). Crystal structures of the μ receptor in the inactive form²⁸ (RSCB PDB code 4DKL) and the active form³¹ (RSCB PDB code 5C1M) were obtained from the RSCB PDB 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.⁴⁰ 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.

In Vitro Biological Evaluation

Opioid Receptor Binding Assays—Opioid receptor binding studies were performed as described elsewhere.⁴¹ Binding affinities for μ and δ receptors were determined by displacing, respectively, [³H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [³H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor binding affinities were measured by displacement of tritiated (5α,7α,8β-(–)-*N*-methyl-*N*-[7-(1-pyrrolidinyl-1-oxaspiro[4.5]dec-8-yl)]benzeneacetamide ([³H]U69,593)⁴² (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 0.80 nM. IC₅₀ values were determined from log dose–displacement

curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,⁴³ using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [³H]DAMGO, [³H]DSLET, and [³H]U69,593, respectively.

Functional Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays—

The GPI⁴⁴ and MVD⁴⁵ functional assays were carried out as reported in detail elsewhere.^{41,46} A dose–response curve was determined with [Leu⁵]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.⁴⁷ K_e values for antagonists were determined from the ratios of IC_{50} values obtained with an agonist in the presence and absence of a fixed antagonist concentration.⁴⁸ μ antagonist K_e values were determined in the GPI assay against the μ agonist TAPP (H-Tyr-D-Ala-Phe-Phe-NH₂)⁴⁹ and in some cases also against the μ agonists morphine (for compounds **1** and **2**), [Dmt¹]DALDA (for compound **1**), and fentanyl (for compound **1**). κ antagonist K_e values of compounds were also determined in the GPI assay against the κ agonist U50,488 and for compounds **1** and **2** also against dynorphin A(1–13).

Plasma Stability Assay—Stability experiments were performed with rat plasma (Sigma-Aldrich) using a published protocol.⁵⁰ After defrosting the plasma at room temperature solids were removed by centrifugation. Peptides were dissolved as a 0.5 mg/mL solution in 0.01 M phosphate buffer (pH 7.4). An amount of 50 μ L of the peptide solutions was transferred into Eppendorf tubes, and 50 μ L of rat plasma supernatant was added. After mixing, the samples were incubated at 37 °C. Samples were analyzed initially and then after 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 3 h, 24 h, and 48 h. The samples were quenched with 50 μ L of acetonitrile and 400 μ L of 2% TFA/water. After vortexing, the quenched samples were analyzed by reversed-phase HPLC on a Vydac C18 column (4.6 mm $\times$ 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. Peptide peak areas were integrated, and the percent peptide left compared to the initial was graphed against time.

Determination of Serum Albumin Binding—A solution of bovine serum albumin (BSA) (20 mg/mL) in 0.01 M phosphate buffer (pH 7.4) was prepared. Peptides (1 mg) were dissolved in 1 mL of the BSA solution. As comparative samples, peptides (1 mg) were dissolved in 1 mL of 0.01 M phosphate buffer (pH 7.4) only. Samples were incubated at 37 °C for 24 h, 48 h, and 96 h, and an aliquot of the samples was analyzed by reversed-phase HPLC on a Vydac C18 column (4.6 mm $\times$ 250 mm) with a linear gradient of 20% MeOH in 0.1% TFA over 30 min at a flow rate of 1 mL/min. No decrease in peptide peak intensity was observed with the samples of peptides **1** and **9**, indicating that BSA adsorption did not occur.

In Vivo Brain Uptake Study

Male CD1 mice (27 g each) were given intravenous injections of 2 mg/kg of **1** or **9** dissolved in saline. Brain and plasma samples were taken at 5, 15, 30, 60, 120, and 240 min and frozen on ice. The brain samples were weighed frozen and homogenized in 1 mL of phosphate buffer saline (PBS, pH 7.4). Samples were then centrifuged, and the supernatant was dried

over nitrogen and reconstituted in an equal volume of methanol. Plasma proteins were precipitated, and 4× the plasma volume of acetonitrile/methanol (1:1) was added. After standing for 30 min on ice, the samples were centrifuged and the supernatant was dried under nitrogen and reconstituted in an equal volume of methanol. Internal standards were added as follows: compound **9** for analysis of compound **1** and compound **1** for analysis of compound **9**. Peptides were quantitated by LC–MS/MS analysis. Stock solutions of 1 µg/mL of each peptide in water were made fresh for each assay and diluted in water to concentrations in the range of 0.01 ng/mL to 50 ng/mL. Standard curves were also made in mouse brain homogenate and extracted as described for the samples. Each standard curve was generated using linear regression weighted to 1/x. Unknowns were calculated using the ratio of the compound and internal standard from the appropriate extracted tissue standard curve. An Acella HPLC instrument was the front end for an ABI 4000 Qtrap mass spectrometer. The mobile phases used were water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) in a gradient elution starting at 95% A, transitioning in a linear gradient to 5% over 30 s and held for 5 min before returning to initial conditions. Samples of 10 µL each were injected onto an Agilent stable bond C8 column (2 mm × 50 mm, 5 µm) with a C8 guard column. For peptides **1** and **9** the brain AUCs were 0.052 µg·min/g and 0.352 µg·min/g, respectively (Figure 7), and the plasma AUCs were 0.0303 µg·min/mL and 4.98 µg·min/mL, respectively.

Evaluation of MOR Antagonist Activity in Analgesic Tests

Tail Heat Algesia Testing—Tail withdrawal latency (TWL) tests were performed with Sprague-Dawley rats (250–650 g). Each rat was placed in a closed acrylic enclosure on the glass surface of a Hargreaves apparatus (no. 390G, IITC Life Science, Woodland Hills, CA). A point 5–10 cm from the tip of the tail was subjected to irradiation by a focused light beam approximately 4 mm × 6 mm in area. The time to tail withdrawal from the light was recorded and the light switched off. The intensity of the light had been set previously to produce a tail flick within approximately 8 s of stimulation (as previously determined in other animals, data not shown). In the absence of a response, a cut-off time of 20 s was applied to each stimulation period. Each measurement during the course of any session consisted of the average of three tests, given at intervals of 5 min.

Hot Plate Algesia Testing—Hot plate algesia testing was always performed after the TWL test. Forepaw heat withdrawal thresholds (HWTs) were measured using an incremental heating apparatus (no. PE34 incremental hot plate, IITC Life Sciences, Woodland Hills, CA). Each rat was placed on a metal plate inside an acrylic enclosure (10.5 cm wide × 180 cm long × 150 cm high) and preheated to 35 °C and heated to a maximum, cut-off temperature of 50 °C over a 2 min trial. The trial ended with the first forepaw withdrawal and licking or when the cut-off temperature was reached.

Pharmacological Treatments—A total of 21 animals were used for the drug trials. Drugs were tested for their effects on TWL and HWT in separate tests given morphine 4.0 mg/kg sc (as morphine sulfate) ($n = 18$), a combination of morphine 4.0 mg/kg sc followed 20 min later by cyclodal at 1.0 or 5.0 mg/kg iv ($n = 6$ and $n = 9$, respectively). In addition, the nonselective antagonist naloxone hydrochloride (Tocris Biochemicals, Bristol, U.K.) was

administered at a dose of 1.0 mg/kg iv 15 min following morphine to a set of seven animals. The same drugs and combinations were tested for their effects on TWL and HWT. Rats were tested in groups of three or four in order to allow the measurement of both TWL and HWT in the same animal at time intervals of 20 min. After a 20 min period of acclimatization, baseline measurements were obtained for TWL and then HWT before morphine and drug injections and 30, 60, 90, and 120 min after administration of morphine.

Data Analysis—For each drug (and drug combination), TWLs and HWTs were averaged by dose and treatment time and subjected to analysis of variance (ANOVA) using repeated measures. Drug effects were then compared between drug treatments at each testing time point by pairwise comparisons using Tukey's test when a significant time by drug interaction was observed. Statistical analyses were performed using Statistica 6 (StatSoft, Tulsa, OK) and GraphPad Prism (version 5, GraphPad Software, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The work was supported by grants from the National Institute on Drug Abuse, NIH (Grant DA004443 to P.W.S. and Grant DA032020 to J.G.), the Canadian Institutes of Health Research (Grant MOP-89716 to P.W.S. and Grant MOP-119279 to T.J.C.), and the National Sciences and Engineering Research Council of Canada (Grant RGPIN 194521 to T.J.C.).

ABBREVIATIONS USED

D-A₂bu	D- α , γ -diaminobutyric acid
BBB	blood–brain barrier
BOP	benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate
D-Cit	D-citrulline
6-Cl-HOBt	6-chloro- <i>N</i> -hydroxybenzotriazole
CTOP	H-D-Phe-c[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH ₂
cyclodal	c[-D-Arg-Phe-Lys-Dmt-]
DAMGO	H-Tyr-D-Ala-Gly-N ^α MePhe-Gly-ol
DEA	diethylamine
DIC	<i>N,N</i> -dicyclohexylcarbodiimide
DIEA	diisopropylethylamine
Dmt	2',6'-dimethyltyrosine
[Dmt¹]DALDA	H-Dmt-D-Arg-Phe-Lys-NH ₂

DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
Et₂O	diethyl ether
β-FNA	β-funaltrexamine
GPI	guinea pig ileum
D-hArg	D-homoarginine
βhLys	β-homolysine
HWT	heat withdrawal threshold
MVD	mouse vas deferens
Nle	norleucine
Orn	ornithine
D-Pen	D-penicillamine
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
sc	subcutaneous
SPPS	solid phase peptide synthesis
TAPP	H-Tyr-D-Ala-Phe-Phe-NH ₂
TEA	triethylamine
TMH	transmembrane helix
TWL	tail withdrawal latency
Z	benzyloxycarbonyl

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Figure 1.

Chemical structures of c[D-Arg-Phe-Lys-Dmt-] (**1**), c[-Arg-D-Phe-D-Lys-D-Dmt-] (**2**), and c[-D-Arg-Phe-LysΨ(CH₂NH)Dmt-] (**8**).

Figure 2. Receptor-bound structures of the μ opioid antagonist c[-D-Arg-Phe-Lys-Dmt-] (**1**) (magenta) and the μ opioid agonist c[-D-Arg-Phe-Lys Ψ [CH₂NH]Dmt-] (**8**) (cyan) with lowest-energy all-*trans* peptide backbone conformations.

Figure 3.

Docking of c[-D-Arg-Phe-Lys-Dmt-] (1) (magenta) to the crystal structure of the MOR in the inactive state (RSCB PDB code 4DKL).²⁸ MOR-bound β -FNA (crystal structure)²⁸ is depicted in green. MOR key residues: Asp¹⁴⁷, Glu²²⁹ (depicted in red); His²⁹⁷ (depicted in blue).

Figure 4. Docking of c[-D-Arg-Phe-Lys-Dmt-] (**1**) (magenta) and of its optical antipode c[-Arg-D-Phe-D-Lys-D-Dmt-] (**2**) (green) to the crystal structure of the MOR in the inactive state (RSCB PDB code 4DKL). MOR key residues: Asp¹⁴⁷, Glu²²⁹ (depicted in red); His²⁹⁷ (depicted in blue).

Figure 5.

Docking of c-[$\text{D-Arg-Phe-Lys}\Psi[\text{CH}_2\text{NH}]\text{Dmt-}$] (**8**) (magenta, with reduced peptide bond depicted in orange) to the crystal structure of the MOR in the active state (RSCB PDB code 5C1M).³¹ MOR-bound Bu72 (crystal structure)³¹ is depicted in cyan. MOR key residues: Asp¹⁴⁷, Glu²²⁹ (depicted in red); His²⁹⁷ (depicted in blue).

Figure 6. Stability of [Dmt¹]DALDA (●), cyclodal (■), CTOP (▼), and [Leu⁵]enkephalin (▲) in rat plasma.

Figure 7. Brain uptake of [Dmt¹]DALDA (▲) and cyclodal (■). Peptides were administered iv to male CD1 mice.

Figure 8.

Antagonism by cyclodal and naloxone of morphine effects on tail withdrawal latency (TWL) assayed by the Hargreaves test for tail heat algesia (A) and on forepaw heat withdrawal threshold (HWT) assayed by hot plate algesia test (B). Asterisks denote the presence of a significant difference between treatment and vehicle at the same time point ((*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$). Daggers denote the presence of a significant difference between treatment and morphine at the same time point ((†) $P < 0.05$, (††) $P < 0.01$, (†††) $P < 0.001$).

Scheme 1.
Synthesis of Cyclic Peptide 1

Scheme 2.
Synthesis of Cyclic Peptide 5

Scheme 3.
Synthesis of Cyclic Peptide 8

Table 1 Opioid Receptor Binding Affinities of Cyclic [Dmt¹]DALDA Analogues^a

compd	peptide	K_i^{μ} (nM)	K_i^{δ} (nM)	K_i^{κ} (nM)	K_i ratio $\mu/\delta/\kappa$
1	c[-D-Arg-Phe-Lys-Dmt-]	0.0637 $\pm$ 0.0047	1.53 $\pm$ 0.08	5.11 $\pm$ 0.43	1/24/80
2	c[-Arg-D-Phe-D-Lys-D-Dmt-]	1.38 $\pm$ 0.19	263 $\pm$ 6	405 $\pm$ 84	1/191/293
3	c[-D-Cit-Phe-Lys-Dmt-]	8.12 $\pm$ 0.40	425 $\pm$ 85	1340 $\pm$ 180	1/52/165
4	c[-D-Arg-Phe-Nle-Dmt-]	1.54 $\pm$ 0.18	15.0 $\pm$ 2.8	22.7 $\pm$ 5.3	1/10/15
5	c[-D-hArg-Phe-Lys-Dmt-]	0.0323 $\pm$ 0.0008	0.807 $\pm$ 0.200	0.733 $\pm$ 0.122	1/25/23
6	c[c]-D-Arg-Phe-Orn-Dmt-]	0.0745 $\pm$ 0.0095	3.84 $\pm$ 0.01	4.55 $\pm$ 0.40	1/52/61
7	c[-D-Arg-Phe- β -hLys-Dmt-]	0.124 $\pm$ 0.009	16.7 $\pm$ 0.4	0.786 $\pm$ 0.010	1/135/6
8	c[-D-Arg-Phe-Lys- Ψ [CH ₂ NH]Dmt-]	9.02 $\pm$ 1.51	422 $\pm$ 107	60.9 $\pm$ 0.6	1/47/7
9	H-Dmt-D-Arg-Phe-Lys-NH ₂	0.143 $\pm$ 0.015	2100 $\pm$ 310	22.3 $\pm$ 4.2	1/14700/156
10	CTOP	1.30 $\pm$ 0.23	258 $\pm$ 59	14900 $\pm$ 500	1/198/11500

^aMean of three to four determinations $\pm$ SEM.

Table 2 Opioid Antagonist and Agonist Activities of Cyclic [Dmt¹]DALDA Analogues^a

compd	peptide	GPI		IC ₅₀ (nM)	MVD, ^d IC ₅₀ (nM)
		K _e ^μ (nM) ^b	K _e ^κ (nM) ^c		
1	c[-D-Arg-Phe-Lys-Dmt-]	3.26 ± 0.84	40.3 ± 2.6		PA
2	c[-Arg-D-Phe-D-Lys-D-Dmt-]	83.7 ± 7.2	1320 ± 100		PA
3	c[-D-Cit-Phe-Lys-Dmt-]	402 ± 14	307 ± 29		2050 ± 10
4	c[-D-Arg-Phe-Nle-Dmt-]	15.1 ± 2.9	49.7 ± 3.9		PA
5	c[-D-hArg-Phe-Lys-Dmt-]	5.16 ± 1.24	25.4 ± 5.1		PA
6	c[-D-Arg-Phe-Orn-Dmt-]	16.8 ± 2.6	265 ± 55		PA
7	c[-D-Arg-Phe-β-hLys-Dmt-]	10.5 ± 1.1	18.2 ± 1.7		inactive
8	c[-D-Arg-Phe-Lys-Ψ[CH ₂ NH]Dmt-]			2510 ± 360	PA
9	H-Dmt-D-Arg-Phe-Lys-NH ₂			1.41 ± 0.29	23.1 ± 2.0
10	CTOP	40.9 ± 2.7	6940 ± 580		PA

^aMean of three to four determinations ± SEM.^bDetermined against TAPP.^cDetermined against U50,488.^dPA = partial agonist.