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New endomorphin analogs with μ-agonist and δ-antagonist properties*

G. Tóth[1,‡], A. Keresztes[1], Cs. Tömböly[1], A. Péter[2], F. Fülöp[2], D. Tourwé[3], E. Navratilova[4], É. Varga[4], W. R. Roeske[4], H. I. Yamamura[4], M. Szűcs[1], and A. Borsodi[1]

[1]Institute of Biochemistry, Biological Research Center of Hungarian Academy of Sciences, Szeged, Hungary; [2]University of Szeged, Hungary; [3]Department of Organic Chemistry, Vrije University, Brussels, Belgium; [4]University of Arizona Health Science Center, Tucson, AZ 85724, USA

Abstract: Endomorphins (endomorphin-1, H-Tyr-Pro-Trp-Phe-NH_2, endomorphin-2, Tyr-Pro-Phe-Phe-NH_2) are potent and selective μ-opioid receptor agonists. In order to improve the affinity and chemical stability of endomorphins, we have designed, synthesized, and characterized novel analogs with unnatural (2′, 6′-dimethyltyrosine, Dmt) and/or β-alicyclic amino acids (ACPC and ACHC). Radioligand binding assay indicated that several of the novel analogs exhibit high affinity for both μ- and δ-opioid receptors in rat- or mouse-brain membrane preparations. The most promising derivatives—such as Dmt-Pro-Trp/Phe-Phe-NH_2, Dmt-(1*S*,2*R*)-ACPC-Phe-Phe-NH_2, and Dmt-(1*S*,2*R*)-ACHC-Phe-Phe-NH_2)—were characterized in recombinant cell lines expressing human μ- or δ-opioid receptors. Interestingly, while these novel peptides were potent opioid agonists in the functional [^{35}S]GTPγS binding assays in Chinese hamster ovary cells expressing the μ-opioid receptors, some behaved as antagonist or inverse agonist in the human δ-opioid receptor-expressing CHO cells. Since it has previously been demonstrated that the coadministration of δ-antagonists with μ-analgesics attenuates the development of analgesic tolerance, introduction of high-affinity δ-antagonist properties into the μ-agonist endomorphins is expected to lead to potent analgesics that produce limited tolerance.

INTRODUCTION

Two new endogenous peptides, endomorphin-1 and endomorphin-2, have recently been isolated from bovine and human brain; their tetrapeptide sequences differ somewhat from those of the classical opioid peptides. These peptides activate μ-opioid receptors with high affinity and selectivity relative to δ- and κ-opioid systems [1,2]. Their Pro residue plays an important role as compared with the other amino acids in peptides as it is the only residue leading to a dialkyl or tertiary amide bond when incorporated by a ribosomal mechanism into a polypeptide. This chemical difference from other amino acids leads to a low energy barrier between the *cis* and *trans* isomers of an amide bond preceding a Pro residue. The difference in free energies between the two forms has been estimated at 2–4 kcal/mol [3]. The endomorphins therefore exist in *cis* and *trans* forms with respect to the Tyr-Pro peptide bond [4]. ^{1}H NMR spectroscopy studies and molecular dynamics calculations support *cis/trans* equilibrium with a *trans* isomer predominance (*trans/cis* ratio = 2:1) [5,6]. However, conformational studies of these

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‡Corresponding author

peptides in solution do not permit definitive conclusions with regard to the receptor-bound conformation, even if they indicate that one isomer is predominant [7]. *Cis/trans* isomerization is a dynamic process, and the two isomers cannot be isolated at room temperature. Accordingly, direct experimental determination of the conformation of a receptor-bound peptide is not yet feasible and there are no direct binding data on the *cis* or *trans* peptide isomers.

Despite the structural diversity of the opioid peptides, the *N*-terminal Tyr is a common structural element. The Tyr is relatively intolerant to chemical modifications [8,9]. The only exception is alkylation of its aromatic ring. Replacement of the Tyr for Dmt in the opioid peptides generally results in a higher binding affinity and bioactivity of opioid agonists and antagonists [10]. Methyl groups can attribute the beneficial effect of Dmt to the reduction of acidity of the phenolic hydroxyl group due to the electron-donating methyl substituents and the optimalization of the hydrophobic and steric properties of the peptide.

The opioid peptides in general have limited in vivo half-life because they are prone to proteolytic degradation. The endomorphins are more stable against proteolytic enzymes compared to enkephalins and dynorphins; however, several peptidomimetics or peptide analogs with β-amino acids lead to even more stable endomorphins. Endomorphins containing β-Pro display enhanced enzymatic resistance to enzymatic hydrolysis [11]. In recent years, a number of investigations have been performed to introduce alicyclic β-amino acids such as *cis*- and *trans*-2-aminocyclopentanecarboxylic acid (ACPC) and *cis*- and *trans*-2-aminocyclohexanecarboxylic acid (ACHC) into the peptides in order to increase their enzymatic stability and to modify their biological activity [12]. An example was morphiceptin where ACPC was used as a proline mimetic [13]. β-Peptides, formed by β-amino acids having an extra backbone carbon, have been intensively studied in a search for stable, well-ordered secondary structures [14].

This presentation reports on novel endomorphin analogs with Dmt and alicyclic β-amino acids (ACPC, ACHC) as Pro mimetics to estimate bioactive conformation, in conjunction with classical SAR studies and to improve their affinity and proteolytic stability.

RESULTS AND DISCUSSION

The new endomorphin analogs were synthesized by solid-phase peptide synthesis (SPPS) using L-Dmt and alicyclic β-amino acids (Fig. 1). The protected derivatives of the *cis* (1*S*,2*R*) and (1*R*,2*S*) isomers and the *trans* (1*S*,2*S*) and (1*R*,2*R*) isomers (Fig. 2) were prepared in our laboratory by literature methods [12,15]. Chirally pure L-Dmt was obtained from racemic *N*-trifluoroacetyl-Dmt by enzymatic treat-

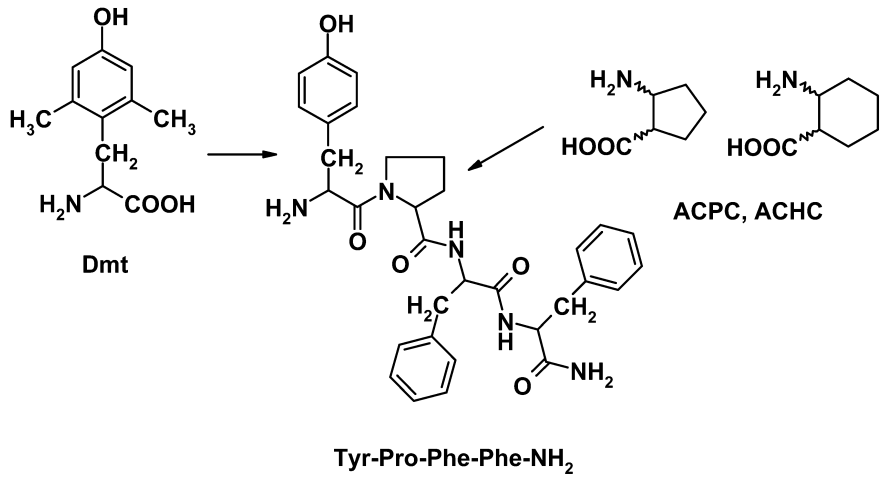

Fig. 1 Structural modifications of endomorphin-2.

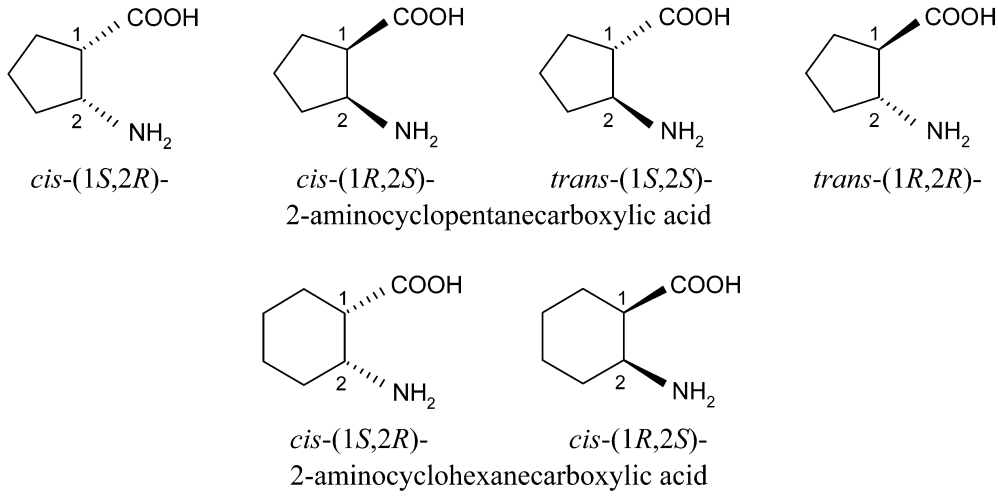

Fig. 2 Alicyclic β-amino acids.

ment with carboxypeptidase A. The ACPC- and ACHC-containing epimeric peptides were separated by HPLC. The configuration of each alicyclic β-amino acid in the peptides was determined after acidic hydrolysis and GITC derivatization [16] with the use of chirally pure standards for comparison. Enantiopure β-amino acids were prepared by biocatalysis using lipases and *N*-hydroxymethylated β-lactam derivatives [17].

The biological properties of the endomorphin analogs were determined in radioligand binding assays based on the displacement of tritiated opioid ligands in rat- and mouse-brain membranes (Tables 1–3). The binding affinities of Dmt[1]-endomorphin-1 and Dmt[1]-endomorphin-2 were very high (subnanomolar range) on μ-opioid receptors, but they bind to the δ-opioid receptors too, indicating that introduction of a Dmt moiety reduces the selectivity of the new analogs. Dmt[1]-endomorphin-2 has also been prepared and characterized by Okada and coworkers [18], and our data have confirmed their results.

Table 1 Binding affinities of endomorphins and Dmt[1]-endomorphins in rat-brain membrane preparation.

Peptides	$K_{i\mu}$[a] (nM)	$K_{i\mu}$[b] (nM)	$K_{i\delta}$[c] (nM)	$K_{i\delta}$[d]/$K_{i\mu}$	Na-index[e]
Tyr-Pro-Trp-Phe-NH_2	1.62 ± 0.12	4.21 ± 0.31	6390 ± 5390	1517	30.5
Dmt-Pro-Trp-Phe-NH_2	0.005 ± 0.001	0.014 ± 0.003	12.0 ± 4,05	857	13.8
Tyr-Pro-Phe-Phe-NH_2	4.00 ± 1.22	9.53 ± 2.19	2650 ± 1750	662	30.4
Dmt-Pro-Phe-Phe-NH_2	0.039 ± 0.016	0.205 ± 0.085	93.6 ± 16	456	6.9

a[3H]-Endomorphin-2.
b[^{3}H]-DAMGO.
c[^{3}H]-Ile^{5,6}-deltorphin-2.
d$K_{i\delta}/Ki_{\mu}$, selectivity ratio: inhibition constant K_i of the peptides against [^{3}H]-DAMGO over K_i against [^{3}H]-Ile^{5,6}-deltorphin-2.
eSodium index: inhibition constant of the peptides against [^{3}H]-naloxone in the presence over in the absence of Na^+ ($K_{i\ Nx+Na+}/K_{i\ Nx}$).
The values in the table are means ± SD.

Table 2 Binding affinities of new endomorphins containing ACPC or ACHC in rat-brain membrane preparation.

Peptides	$K_{i\mu}$ [a] (nM)	$K_{i\delta}$ [b] (nM)	$K_{i\delta}/K_{i\mu}$ [c]
Endomorphin-1	2.1 ± 0.6	6390 ± 539	3044
[(1*S*,2*R*)-ACPC]^2-endomorphin-1	6.29 ± 1.26	>1000	>1590
[(1*R*,2*S*)-ACPC]^2-endomorphin-1	196 ± 32	>1000	>51
[(1*S*,2*R*)-ACHC]^2-endomorphin-1	1.5 ± 0.3	2763 ± 213	1842
[(1*R*,2*S*)-ACHC]^2-endomorphin-1	467 ± 103	2839 ± 289	6.0
Endomorphin-2	1.3 ± 0.16	2650 ± 175	2038
[(1*S*,2*R*)-ACPC]^2-endomorphin-2	0.72 ± 0.16	2625 ± 609	3646
[(1*R*,2*S*)-ACPC]^2-endomorphin-2	2049 ± 831	7059 ± 1216	3.4
[(1*S*,2*R*)-ACHC]^2-endomorphin-2	0.5 ± 0.1	223 ± 57	446
[(1*R*,2*S*)-ACHC]^2-endomorphin-2	263 ± 66	2074 ± 482	7.8

[a][^{3}H]-Endomorphin-2.
[b][^{3}H]-Ile^{5,6}-deltorphin-2.
[c]Selectivity.

Table 3 Binding affinities of the endomorphin analogs with double (Dmt^1-ACPC/ACHC^2) modifications in mouse-brain membrane preparations.

Peptides	K_i [a] (nM)	K_i [b] (nM)
Tyr-Pro-Trp-Phe-NH_2	7.2	>1000
Tyr-Pro-Phe-Phe-NH_2	21.0	1070
Dmt-Pro-Trp-Phe-NH_2	0.4	9
Dmt-Pro-Phe-Phe-NH_2	0.6	145
Tyr-(1*S*,2*R*)-ACPC-Phe-Phe-NH_2	14.6	2460
Dmt-(1*S*,2*R*)-ACPC-Phe-Phe-NH_2	1.2	55
Dmt-(1*R*,2*S*)-ACPC-Phe-Phe-NH_2	28	550
Dmt-(1*S*,2*R*)-ACHC-Phe-Phe-NH_2	2.7	57
Dmt-(1*R*,2*S*)ACHC-Phe-Phe-NH_2	980	>1600

[a][^{3}H]-DAMGO.
[b][^{3}H]-Deltorphin-2.

In order to determine the potencies and intrinsic activities of novel compounds, [^{35}S]GTPγS binding assays have also been performed using rat-brain and recombinant CHO cell membrane preparation. Both Dmt^1-endomorphin analogs behaved as partial agonist in rat-brain membrane preparations (data not shown). A partion agonist property was revealed by the Na index of Dmt^1-endomorphin-2 with a value of 6.9 (see Table 1). The Dmt^1-endomorphins also concentration-dependently stimulate [^{35}S]GTPγS binding to hMOR/CHO cell membrane, with E_{max} values similar to that of DAMGO as a standard, indicating that the peptides are full agonist on the μ-opioid receptors (Table 4). Conversely, the Dmt^1-endomorphins did not stimulate [^{35}S]GTPγS binding to hDOR/CHO cell membrane, indicating that the analogs may act as antagonist at δ-opioid receptors. In collaboration with Schiller and Tourwé, we earlier prepared some mixed μ-agonist/δ-antagonist TIPP analogs [19,20] starting from the δ-antagonist compound, Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2). It was demonstrated that coadministration of a μ-agonist and a δ-antagonist reduces the development of tolerance [19]. Introduction of high-affinity δ-antagonist properties into the μ-agonist endomorphins is, therefore, expected to yield potent analgesics that produce limited tolerance.

Table 4 Stimulation of [^{35}S]GTPγS binding by selected endomorphin derivatives in hDOR/CHO or hMOR/CHO cell membranes.

Peptides	EC_{50} (nM) (hDOR/CHO)	E_{max} (% above/below basal hDOR/CHO)	EC_{50} (nM) (hMOR/CHO)	E_{max} (% above/below basal hMOR/CHO)
Dmt-Pro-Trp-Phe-NH_2	7.8	–3 ± 7	2	104 ± 2
Dmt-Pro-Phe-Phe-NH_2	40	–14 ± 10	2	130 ± 2
Dmt-(1*S*,2*R*)-ACPC-Trp-Phe-NH_2	94 ± 6	68 ± 24	11	360 ± 2
Dmt-(1*S*,2*R*)-ACHC-Phe-Phe-NH_2	38 ± 1	56 ± 4	4	400 ± 7

In radioligand binding assays we found that the endomorphin analogs containing (1*S*,2*R*)-alicyclic β-amino acids had higher affinities than the (1*R*,2*S*) analogs at μ-opioid receptors (Table 3). The endomorphins with *trans*-(1*S*,2*S*)-ACPC or (1*S*,2*S*)-ACHC also exhibited lower affinities than the (1*S*,2*R*) analogs (data not shown). The binding affinities of the new analogs at δ-opioid receptors were much lower. Some analogs—(1*S*,2*R*)-$ACPC^2$-endomorphin-1, (1*S*,2*R*)-$ACHC^2$-endomorphin-1, and (1*S*,2*R*)-$ACPC^2$-endomorphin-2—were very selective for μ-opioid receptors. These analogs proved more active than the parent peptides in the displacement binding assay with [^{3}H]-endomorphin-2 as radioligand [21].

The promising results obtained using the Dmt^1-endomorphins and endomorphins with alicyclic β-amino acids in position 2 encouraged us to investigate the combined effects of Dmt^1 and *cis*-ACPC or *cis*-ACHC substitution in endomorphins. The new doubly modified analogs were synthesized and tested in radioligand binding assay using mouse brain (Table 3) and [^{35}S]GTPγS binding using recombinant hDOR/CHO and hMOR/CHO cells (Table 4). Both Dmt^1-(1*S*,2*R*)-$ACPC^2$/$ACHC^2$-endomorphin-2 exhibited high affinity for μ-opioid receptor; and both behaved full agonist in the [^{35}S]GTPγS binding assay in recombinant hMOR/CHO cell membranes. Interestingly, however, despite its high affinity to inhibit the binding of [^{3}H]-deltorphin-2 to mouse-brain membranes, Dmt^1-(1*S*,2*R*)-$ACPC^2$-endomorphin-2 was not able to stimulate [^{35}S]GTPγS binding to recombinant hDOR/CHO cell membranes, indicating that the compound may have δ-antagonist properties.

Previous systematic studies demonstrated that endomorphin-1 and endomorphin-2 in a rat-brain homogenate were degraded slowly by proteolytic enzymes, which first cleave the Pro-Trp or Pro-Phe peptide bonds [22]. In the present study, we investigated the stabilities of the new endomorphins substituted with Dmt in position 1 and alicyclic β-amino acids in position 2 by using HPLC technique. The incorporation of (1*S*,2*R*)-ACPC or (1*S*,2*R*)-ACHC instead of Pro in the endomorphins resulted in practically enzyme-resistant peptides (half-lives of the endomorphin derivatives were >12 h, in rat-brain homogenate at 37 °C, pH 7.4) (Table 5).

Table 5 Half-lives of the endomorphin analogs in rat-brain membrane.

Peptides	Half-life
Tyr-Pro-Trp-Phe-NH_2	4.94 ± 0.19 min
Dmt-Pro-Trp-Phe-NH_2	123.34 ± 4.75 min
Tyr- (1*S*,2*R*)-ACPC-Trp-Phe-NH_2	>12 h
Tyr-(1*S*,2*R*)-ACHC-Trp-Phe-NH_2	>12 h
Tyr-Pro-Phe-Phe-NH_2	3.81 ± 0.19 min
Dmt-Pro-Phe-Phe-NH_2	39.55 ± 5.58 min
Try-(1*S*,2*R*)-ACPC-Phe-Phe-NH_2	>12 h
Tyr-(1*S*,2*R*)-ACHC-Phe-Phe-NH_2	>12 h

Values are mean ± SD.

We used the novel analogs to investigate the biologically active, receptor-bound conformation of endomorphins. ^{1}H NMR spectra of endomorphin-2, and Dmt^1-endomorphin-2 in DMSO-d_6 at 300 °K displayed two sets of signals belonging to slowly converting rotamers. The *cis/trans* ratios derived from a conformational equilibrium around the Tyr-Pro, Dmt-Pro, and Tyr-(1*S*,2*R*)-ACPC amide bonds, calculated via integration of the corresponding proton signals, clearly demonstrated differences between the Tyr- and Dmt-containing analogs. Earlier observation [3,18] and our own NMR results [23] revealed that endomorphin-2 prefers the *trans* conformation with a *cis/trans* ratio 1:2; on the other hand, Dmt^1-endomorphin-1 and Dmt^1-endomorphin-2 exist predominantly in the *cis* conformation (the *cis/trans* ratio was 2:1). Incorporation of (1*S*,2*R*)-ACPC as a Pro mimetic in endomorphin-2, the Tyr-(1*S*,2*R*)-ACPC bond contains a normal amide bond, existing only in the *trans* form.

CONCLUSIONS

We have reported the syntheses and the biological activities of a number of analogs of endomorphin-1 and endomorphin-2. Dmt was incorporated in the first position and ACPC and ACHC as Pro mimetics in position 2 of the endomorphins.

The Dmt^1-endomorphins were found to be very active compounds in radioligand binding assays for μ-opioid receptors while they bind to δ-opioid receptors with $K_I < 25$ nM. Interestingly, however, these peptides were full agonists at μ-opioid receptor according to [^{35}S]GTPγS binding assays on hMOR/CHO cells, but they behaved as either δ-antagonists, or inverse agonists on hDOR/CHO cells. It has been demonstrated earlier that δ-antagonists attenuate the development to tolerance to μ-opioid analgesics, we expect that the novel δ-antagonist/μ-agonist endomorphins may prove to be long-acting potent analgesics with a limited propensity for analgesic tolerance.

The chirality of the β-alicyclic amino acids plays an important role in the conformational preferences and in the biological properties of endomorphins. Analogs containing (1*S*,2*R*)-ACPC or (1*S*,2*R*)-ACHC residues were the most active in the radioreceptor assays. Both analogs proved very stable against proteolytic enzymes in a rat-brain membrane homogenate.

The new endomorphin analogs were good tools with which to evaluate the conformation of the Tyr-Pro bonds of endomorphin bound to the opioid receptors. According to literature data [3] and to our NMR and molecular dynamics study [4,5,23], the *cis/trans* ratio in the parent compound was 2:1. In the Dmt^1-endomorphins, the *cis* isomers predominated and were more active than the parent peptides. The *cis/trans* equilibrium in solution does not permit definitive conclusions concerning the receptor-bond conformation of the peptides. The peptides with (1*S*,2*R*)-ACPC in position 2 featured only the *trans* isomer of the Tyr-(1*S*,2*R*)-ACPC bond rotamer. This peptide exhibited similar affinity in radio receptor assay compared to the parent peptide. Finally, from the results of NMR studies, molecular dynamics methods, and different bioassays we suggest a bioactive conformation of endomorphins involving *trans* Tyr-Pro bond.

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