Endomorphin Analogs Containing Alicyclic β-Amino Acids: Influence on Conformation and Pharmacological Profile

Attila Keresztes

Isotope Laboratory, Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences

Supervisors

Dr. Géza Tóth
Dr. Mária Szűcs

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I dedicate

this thesis to
my grandmother, without whom
this work could not have been
achieved.

List of Thesis-Related Publications

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List of Abbreviations

ΔACHC	2-aminocyclohexenecarboxylic acid
ΔACPC	2-aminocyclopentenecarboxylic acid
ACHC	2-aminocyclohexanecarboxylic acid
ACN	acetonitrile
AcOH	acetic acid
ACPC	2-aminocyclopentanecarboxylic acid
BBB	blood brain barrier
B_{max}	maximal number of binding sites
Boc	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
Cl_2Phe	3,4-dichlorophenylalanine
CNS	central nervous system
DAMGO	H-Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol
DCC	N,N-dicyclohexylcarbodiimide
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	dimethylformamide
Dmp	2,6-dimethylphenylalanine
DMSO	dimethyl sulfoxide
Dmt	2,6-dimethyltyrosine
DPDPE	H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH
dynorphin A (1-17)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
EGTA	ethylene glycol tetraacetate
endomorphin-1	H-Tyr-Pro-Trp-Phe-NH_2
endomorphin-2	H-Tyr-Pro-Phe-Phe-NH_2
EtOH	ethanol
GABA	γ-aminobutyric acid

GDP	guanosine diphosphate
GITC	2,3,4,6-*tetra*-*O*-acetyl-glucopyranosyl-isothiocyanate
GPCR	G-protein-coupled receptor
Gpp(NH)p	5'-[β,γ-imido]triphosphate trisodium salt
GTP	guanosine-triphosphate
$GTP_\gamma S$	guanosine-5'-*O*-(3-thio)triphosphate
hDOR	human delta-opioid receptor
Hfe	homophenylalanine
hMOR	human mu-opioid receptor
HOBt	N-hydroxybenzotriazole
HPLC	high performance liquid chromatography
$Ile^{5,6}$-deltorphin-2	H-Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2
K_d	equilibrium dissociation constant
K_i	inhibitory constant
LC	locus coeruleus
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MAPK	mitogen-activated tyrosine kinase
MBHA	4-methylbenzhydrylamine
MD	molecular dynamics
MPE	maximal possible effect
MS	mass spectrometry
1Nal	β-(1-naphthyl)alanine
2Nal	β-(2-naphthyl)alanine
NAc	nucleus accumbens
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NorBNI	norbinaltorphimine
NTI	naltrindole
NTS	nucleus of the solitary tract
PAG	periaqueductal gray
pClPhe	4-chlorophenylalanine

Phg	phenylglycine
POPOP	1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene
PPO	2,5-diphenyloxazole
PTFE	polytetrafluoroethylene
R_f	retention factor
TEA	triethylamine
TFA	trifluoroacetic acid
Tic	1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid
TLC	thin layer chromatography
TM	transmembrane
Tris	tris-(hydroxymethyl)-aminomethane
Tyr-W-MIF	H-Tyr-Pro-Trp-Gly-NH_2
U-50,488H	*trans*-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclo-hexyl]benzeneacetamide
β-MePhe	β-methylphenylalanine

1. Introduction

1.1. The Opioid System

The opioid receptor system is one of the most widely studied families of G-protein-coupled receptors (GPCR), it mediates a large variety of physiological functions and is of considerable importance in pain regulation.

To date, three well-defined types of opioid receptors are known, MOP (μ), DOP (δ) and KOP (κ), which are encoded by unique genes (Martin et al., 1976; Lord et al., 1977). Besides these three major opioid receptor types, many subtypes (μ_1-μ_2, δ_1-δ_2 and κ_1-κ_2-κ_3) have also been suggested on the basis of pharmacological assays, and are thought to be receptor oligomers or different post-translational modifications of the single gene products (Jordan et al., 1999; George et al., 2000).

Structurally, the opioid receptors are composed of a single polypeptide chain with seven transmembrane (TM) domains. These receptors share approximately 60-70% homology with each other, with greatest homology in the TM helices. The TM pockets (TM1, TM2 and TM3) are thought to serve as potential binding sites for opioid ligands, while the 2nd and 3rd intracellular loops have been proposed to interact with $G_{i/o}$-proteins (Harrison et al., 1998). The extracellular N-terminal of the receptor proteins has different numbers of glycosylation sites (Kieffer, 1995). The intracellular C-terminal can be modified post-translationally by lipid acids and contains multiple phosphorylation sites. Both terminals with the extracellular loops have been shown to be highly diverse in amino acid sequences (Chen et al., 1993).

The opioid receptors are expressed in different areas of the central nervous system (CNS), but display region-selective localization and distribution (Mansour et al., 1987). Higher μ-receptor density has been observed for the thalamus, caudate putamen, neocortex and the dorsal horn of the spinal cord (Hawkins et al., 1988). δ-Receptors are abundantly present in the olfactory bulb, neocortex, caudate putamen, nucleus accumbens and amygdala. κ-Receptors display a dense expression in areas of the cerebral cortex, nucleus accumbens and hypothalamus. The peripheral occurrence of the receptors has been also described, thus, they have been shown to be present in great numbers in the smooth muscle, heart, retina and placenta (Mansour et al., 1987).

The opioid receptor family belongs in the G_i/G_o-coupled superfamily of receptors (Harrison et al., 1998). The results of pharmacological and signaling studies strongly suggest that all of the opioid receptors share common effector mechanisms, though subtle differences have been postulated to refer to the downstream effector systems (Buford et al., 1998; Harrison et al., 1999). In general, agonist binding entails a multitude of intracellular events, such as the opening of inwardly rectifying K^+ channels, the closure of definite types (N, P, Q, T and R) of voltage-gated Ca^{2+} channels, the inhibition of adenylyl cyclase, the activation of phospholipase A/C and other kinases (MAPK, mitogen-activated protein kinase) and the inhibition of transmitter release.

The main action of the endogenous and exogenous opioid/opiate ligands is to regulate the nociceptive pathway, including pain perception, modulation and the response to painful stimuli. Many other functions, such as the regulation of the respiratory, cardiovascular, gastrointestinal and immune systems, mood and feeding behavior, and additionally, the development of tolerance and dependence, are known to be concomitant side-effects of opioid administration (Vaccarino et al., 1999; Horvath et al. 2000; Fichna et al., 2007). The mammalian endogenous opioid ligands of the μ-, δ- and κ-receptors (endorphins, enkephalins and dynorphins), and their preproproteins (pro-opiomelanocortin, proenkephalin and prodynorphin) have been thoroughly investigated (Hughes et al., 1975; Li and Chung, 1976; Chavkin et al., 1982). Subsequently, a large number of different opioid receptor-acting peptides, peptide fragments and preproproteins have been isolated and studied from a variety of different species and natural sources. The classification of the endogenous opioid ligands is shown in **Table 1**.

Table 1. Classification of endogenous opioid peptides

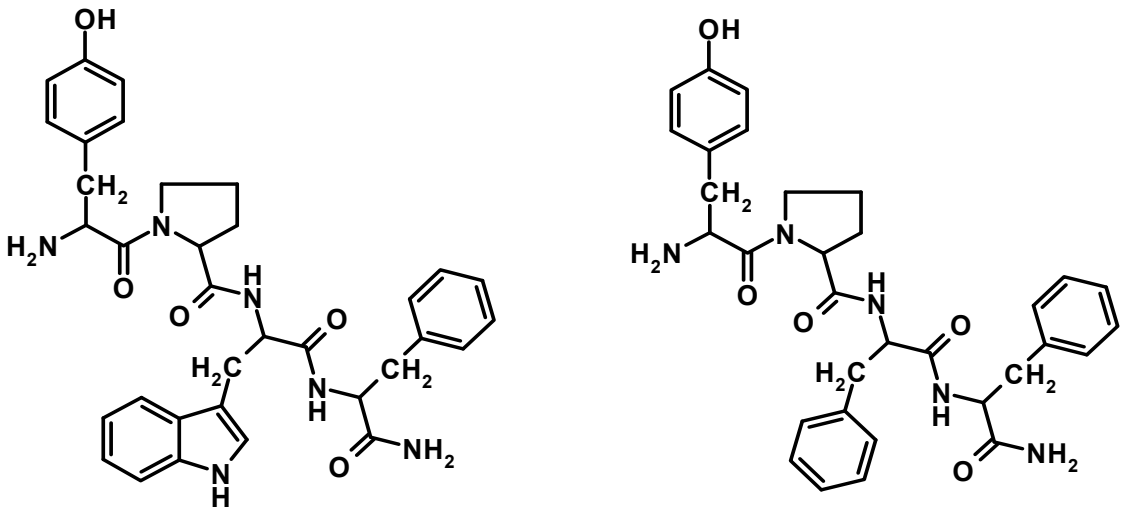
Receptor	Source protein	Peptide fragment	Amino acid sequence	Origin	References
μ (MOR)	Pro-opiomelanocortin	β-Endorphin	YGGFMTSEKQTPLVTLFKN AIIKNAYKKGE	Mammalian brain	*Li and Chung (1976)*
	β-Casein (bovine)	β-Casomorphin-5 β-Casomorphin-7 Morphiceptin	YPFPG YPFPGPI YPFP-NH_2	Bovin milk	*Blanchard et al. (1987)*
	β-Casein (human)	β-Casomorphin-5 β-Casomorphin-7	YPFVE YPFVEPI	Human milk	*Blanchard et al. (1987)*
	Hemoglobin	Hemorphin-4 Hemorphin-7	YPWT YPWTQRF	Human blood	*Nyberg et al. (1997)* *Zhao et al. (1997)*
	Unknown	Dermorphin Endomorphin-1 Endomorphin-2 Tyr-MIF-1 Tyr-W-MIF-1	YaFGYPS-NH_2 YPWF-NH_2 YPFF-NH_2 YPLG-NH_2 YPWG-NH_2	Frog skin Bovine and human brain	*Bozu et al. (1997)* *Zadina et al. (1997)* *Zadina et al. (1994)*
δ (DOR)	Proenkephalin	[Met^5]enkephalin [Leu^5]enkephalin	YGGFM YGGFL	Mammalian brain	*Hughes et al. (1975)*
	Unknown	Dermenkephalin Deltorphin-1 Deltorphin-2	YmFHLMD YaFDVVG-NH_2 YaFEVVG-NH_2	Frog skin	*Amiche et al. (1989)* *Kreil et al. (1989)*
κ (KOR)	Prodynorphin	Dynorphin A Dynorphin A(1-8) Dynorphin B	YGGFLRRIRPKLKWDNNQ YGGFLRRI YGGFLRRQFKVVT	Mammalian brain	*Chavkin et al. (1982)*
ORL_1 (NOP)	Pronociceptin	Nociceptin/orphanin FQ	FGGFTGARKSARKLANQ	Mammalian brain	*Meunier et al. (1995)* *Reinscheid et al. (1995)*

1.2. Discovery of Endomorphins

More than a decade ago, as a result of the pioneering work of Zadina and coworkers, two potent μ-receptor ligands were found when systematic natural amino acid substitution of Tyr-W-MIF (H-Tyr-Pro-Trp-Gly-NH_2) was performed via an amazing combination of combinatorial chemistry and pharmacology (Zadina et al., 1994). Four years later, these tetrapeptides (endomorphins) were isolated first from bovine, and subsequently from human brains, and were named endomorphin-1 (H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2) (Zadina et al., 1997; Hackler et al., 1997). Schematic chemical structures of the endomorphins are shown in **Figure 1**. The endomorphins displayed marked affinities (K_i = 0.3 up to 1.5 nM) and selectivities for the μ-opioid receptor (K_i^{μ}/K_i^{δ},K_i^{κ} = 4,000 and 15,000) in the guinea pig ileum assay and tail-flick tests (Zadina et al., 1997; Hackler et al., 1997), and also interesting pharmacological and structural features. Intracerebroventricular (*i.c.v.*) administration of the endomorphins produced potent antinociceptive effects in wild-type, but not in μ-receptor knock-out mice (Zadina et al., 1999; Narita et al., 1999; Horvath, 2000; Sakurada et. al., 2002). Intrathecal (*i.t.*) administration of the endomorphins evoked significant antinociception in the tail-flick, paw-withdrawal, tail-pressure, and flexor-reflex tests in adult rodents (Stone et al., 1997; Zadina et al., 1997; Goldberg et al., 1998; Horvath et al., 1999; Sakurada et al., 1999, 2000, 2001; Ohsawa et al., 2001; Grass et al., 2002).

1.3. Structural Properties and Modifications of Endomorphins

From a structural point of view, the first notable difference in comparison with the opioid ligands discovered earlier, is their nonconventional sequences, which differ from those of the other endogenous opioid peptides. For the classical opioid peptides, the N-terminal Tyr-Gly-Gly-Phe sequence confers the ”message sequence”, while the remaining C-terminal constitutes the ”address sequence” (Yamazaki et al., 1993; Corbett et al., 1993). In the endomorphins, the ”message sequence” comprises only two pharmacophore amino acids, the Tyr^1 and Trp^3/Phe^3 residues, respectively, which are responsible for the proper binding orientation and receptor recognition (Podlogar et al., 1998,).



Endomorphin-1
(H-Tyr-Pro-Trp-Phe-NH_2)

Endomorphin-2
(H-Tyr-Pro-Phe-Phe-NH_2)

Figure 1. Schematic chemical structures of endomorphins

The Phe^4 and amidated C-terminal (this latter proved to be a free carboxyl function for other endogenous peptides and results in longer half-lives with the Pro^2 residue to endomorphins) encode the "address sequence", which appeared to be essential for the μ-receptor specificity (Yang et al., 1999).

Since the endomorphins possess some disadvantagous characteristics, which render their potential applications difficult for both clinical and research purposes, structural modifications were considered necessary. The endomorphins displayed a short duration of antinociception, and one aim was therefore to prolong their half-lives by substituting the Pro^2 residue with β-, D-Pro and D-Ala amino acids (Champion and Kadowitz, 1998, 1999; Cardillo et al., 2002). Usually, the resulting analogs not only proved to be as potent as endomorphins, but exhibited longer half-lives. Opposite effects were generated when all amino acid residues were randomly substituted with their corresponding D-analogs. The resulting peptides lost their μ-receptor affinities, though their proteolytic stability increased significantly (Paterlini et al., 2000; Okada et al., 2000). Substitution of the Trp^3/Phe^3 residues of the endomorphins with β-(1-

naphthyl)alanine (1Nal), β-(2-naphthyl)alanine (2Nal), 4-chlorophenylalanine (pClPhe), 3,4-dichlorophenylalanine (Cl_2Phe), homophenylalanine (Hfe) or Phenylglycine (Phg) resulted in analogs with decreased binding potencies (Fichna et al., 2005; Gao et al., 2006). Chemical modification of the C-terminal part of the endomorphins, including replacement of the carboxamide group by hydroxymethyl (CH_2-OH), methylester (COOMe) or hydrazine (CO-$NHNH_2$), usually led to similarly potent analogs to the native ligands (Al-Khrasani et al., 2001; Gao et al., 2005). The utilization of β-methylphenylalanine (β-MePhe) in position 4 provided highy potent analogs as described by Tömböly and coworkers (2004). It has been shown that removal of the Phe^4 residues leads to almost complet loss of binding function of the ligands. In general, replacement of Tyr^1 in opioid peptides by other amino acids was shown to be detrimental for retaining the binding activity. Loss of activity was observed in the case of D-Tyr^1 substitution for endomorphin-2 (Okada et al., 2000). Surprisingly, opposite effects were observed when 2,6-dimethyltyrosine (Dmt) and 2,6-dimethylphenylalanine (Dmp) replacements were carried out in the endomorphins (Przewlocki et al., 1999; Sasaki et al., 2003; Tóth et al., 2004). However, Dmt^1 substitution induced an increased affinity not only for the μ-, but also for the δ-opioid receptors, reflecting that Dmt^1 provokes loss of selectivity. N-allylation of Dmt^1-endomorphins readily generates μ-selective antagonists (Li et al., 2007). The combination of Dmt^1 with 1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid (Tic^2) proved useful for the development of μ-agonist/δ-antagonist surrogates, which produced pronounced antinociception with less tolerance than morphine and without the emergence of dependence after chronic administration, even at high doses (Schiller et al., 1999). In addition to the development of endomorphin analogs with high affinity, selectivity and proteolytic stability, chemical modifications were also aimed at elucidating the stereochemical requirements of opioid binding and the bioactive conformations of these relevant tetrapeptides. The above detailed structural modifications are summarized in **Table 2**.

Table 2. Some typical structural modifications performed on endomorphins

Amino acids	Substituted amino acid(s)/modified functional groups	Effect of substitution on endomorphins	References
β-, D-Pro and D-Ala	Pro^2	Longer biological half-lifes	Champion & Kadowitz 1998; 1999 Cardillo et al., 2002
D-Tyr, D-Phe, D-Trp and D-Pro	Tyr^1, Pro^2, Trp^3/Phe^3, Phe^4	Increased proteolytic stability and loss of μ-receptor affinity	Paterlini et al., 2000 Okada et al., 2000
β-(1-naphtyl)alanine (1NaI) β-(2-naphtyl)alanine (2NaI) 4-chlorophenylalanine (pClPhe) 3,4-dichlorophenylalanine (Cl_2Phe) Homophenylalanine (Hfe) Phenylglicine (Phg)	Trp^3/Phe^3	Decreased binding potency	Fichna et al., 2005 Gao et al., 2006
Carboxamide, hydroxymethyl, methylester, hydrazine	C-terminal part (Phe^4)	Similar potency to the parent ligands	Al-Khrasani et al., 2001 Gao et al., 2005
β-methylphenylalanine (β-MePhe)	Phe^4	Increased binding potency	Tömböly et al., 2004
2,6-dimethyltyrosine (Dmt) 2,6-dimethylphenylalanine (Dmp)	Tyr^1	Increased binding potency and decreased receptor selectivity	Przewlocki et al., 1999 Sasaki et al., 2002 Tóth et al., 2002
2,6-dimethyltyrosine (Dmt) and 1,2,3,4-tetrahydroisoquinolinecarboxylic acid (Tic)	Tyr^1 and Pro^2	Increased binding potency and μ-agonist/δ-antagonist property	Schiller et al., 1999
N-allylation of Dmt^1	N-terminal part (Tyr^1)	Generation of μ-receptor antagonist	Li et al., 2007

1.4. Significance of Chirality of Alicyclic β-Amino Acids and Isomerism in Endomorphins

The alicyclic β-amino acids are of a huge importance as a consequence of their unique structural properties (Fülöp 2001). In these amino acids, structurally, the amino and carboxyl functions are located on neighbouring atoms. The adjacent carbon atoms carrying these functional groups are chiral, and their chiral centers are defined as *R* or *S* with a total number of four possible enantiomers (*R,R; R,S; S,S or S,R)*. (The *R/S* abbreviation labels the chiral centers, and is the most important nomenclature system for denoting enantiomers. Its major advantage is that it does not require reference molecule). The incorporation of these racemic molecules into bioactive peptides provides diastereomers. (Diastereomers are stereoisomers that are not related as object and mirror image). The stereo- and regio-isomers, together with the possible ring size expansions and further substitutions, significantly increase the structural diversity of alicyclic β-amino acids (Fülöp 2001). The insertion of an alicyclic β-amino acid into the place of a natural amino acid is currently believed to improve both the biological activity and the proteolytic stability of a peptide.

The endomorphins may exist in *cis*- and *trans*-conformations (Podlogar et al., 1998) and the isomerization around the Tyr^1-Pro^2 omega peptide bond has been extensively studied by means of a great number of different techniques (Podlogar et al. 1998; Fiori et al., 1999; Paterlini et al., 2000; In et al., 2001; Gentilucci et al., 2004; Leitgeb 2007). The considerably low energy barrier (2-4 kcal/mol) existing between the two conformers allows the formation of either the *trans*- or *cis*-conformers with respective population rates (Podlogar et al., 1998; Gentilucci et al., 2004). The majority of findings reported on a 25:75 *cis/trans* ratio with a few exceptions measured in DMSO, water and micelles using ^{1}H-NMR and theoretical calculations (Leitgeb 2007). This *cis/trans* ratio proved to be uniformly valid for both endomorphins.

As concerns the bioactive conformations of the endomorphins, a large number of studies have been carried out, but none provided definitely convincing models for the biologically relevant conformations of peptides (Gentilucci et al., 2004; Leitgeb, 2007). It has been established that the ratio of the two conformers and their extensions strongly

depend on the experimental conditions applied, and easily vary as these are changed, yielding a series of conflicting results (Gentilucci et al. 2004; Leitgeb, 2007). Additionally, endomorphins can adopt compact or extended structures in either the *cis*- or the *trans*-conformation and this further raises the number of possible 3D-structures (Gentilucci et al., 2004; Leitgeb, 2007). Accordingly, the bioactive conformations of the endomorphins remained an unanswered question.

1.5. Stability of Endomorphins

The majority of opioid peptides undergo rapid enzymatic degradation soon after their production. There are many peptide-degrading enzymes, including exo- and endopeptidases and integral membrane-bound or cytosolic peptidases that are able to split off the peptide backbones of the endomorphins (Kato et al., 1978; Dua et al., 1985; Berne et al., 1999; Harbeck et al., 1991). However, due to their unusual sequences, they display the longest proteolytic half-lives (4-5 min.) among the endogenous opioid peptides known so far. Their metabolic pathways have been mapped and the outcomes have provided valuable information for endomorphin engineering (Peter et al., 1999; Tömböly et al., 2002; Janecka et al., 2006; Fujita et al., 2006). *In vivo* and *in vitro* studies showed that mainly two enzymes are responsible for the initiation of the degradation of the endomorphins. The digestion of peptides is triggered by dipeptidyl peptidase IV (a membrane-bound Ser-protease) and carboxypeptidase Y, and then terminated by various aminopeptidases (Shane et al., 1999; Rónai et al., 1999; Sakurada et al., 2003). The total degradation takes approximately 1 and 2-3 h for endomorphin-1 and endomorphin-2, respectively (Péter et al., 1999; Sugimoto-Watanabe et al., 1999). Interestingly, some authors have reported that endomorphin-1 is more resistant to proteolytic degradation than endomorphin-2 *in vivo*, because it produces a longer spinal duration of antinociception (Grass et al., 2002; Fujita et al., 2006). The effects of protease inhibitors on endomorphin degradation have also been studied, and it was shown that the inhibitors (actinonin, diprotin A and Ala-pyrrolidonyl-2-nitrile) may have important roles in delaying the degradation of peptides either *in vivo* or *in vitro* (Spetea et al,. 1998; Sugimoto-Watanabe et al., 1999; Shane et al., 1999; Sakurada et al., 2003). The

degradation products of endomorphin-1 have been examined to elucidate their possible pharmacological effects (Szatmari et al., 2001). The results revealed that the primary degradation products of endomorphin-1 are H-Tyr-Pro-Trp-Phe-OH and H-Pro-Trp-Phe-OH fragments that have negligible affinities for the μ-opioid receptor, do not stimulate G-protein binding and do not dispose of antinociceptive potencies.

1.6. Neuroanatomical Localization of Endomorphins

Immunofluorescent and immunocytochemical assays performed in rodent CNS have revealed that the endomorphins are widely distributed in the CNS, but they display pronounced differences in anatomical localization. Both endomorphins are abundantly present in areas of the stria terminalis, the periaqueductal gray (PAG), the locus coeruleus (LC), the parabrachial nucleus and the nucleus tractus solitarii (NTS) (Hackler et al., 1997; Martin-Schild et al., 1997, 1999; Pierce et al., 1998; Schreff et al., 1998; Pierce and Wessendorf, 2000). However, the density of endomorphin-1 labeling was found to be higher in areas of the central and peripheral nervous system, the upper brainstem, and particularly in the nucleus accumbens (NAc), cortex, amygdala, thalamus, hypothalamus, striatum and dorsal root ganglia (Schreff et al., 1998; Martin-Schild et al., 1999; Pierce and Wessendorf, 2000). In contrast, endomorphin-2 proved to be more prevalent in the spinal cord and the lower brainstem (Martin-Schild et al., 1999; Pierce and Wesserdorf, 2000). The endomorphins are also present in significant amounts in the peripheral tissues of the spleen, thymus and blood, and interestingly, even in immune cells, where they are presumed to originate from nerve fibers and terminals of the spinal cord (Jessop et al., 2000; Mousa et al., 2002). Receptor autoradiography studies have confirmed that the endomorphins act upon the μ-opioid receptors, as determined by the distribution of the labeling, which agreed well with that measured with the prototypic μ-receptor-specific DAMGO (H-Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) (Goldberg et al., 1998; Kakizawa et al., 1998; Sim et al., 1998). The distribution of endomorphin-2 labeling was identical to that of the μ-receptor ligand β-endorphin (Finley et al., 1981; Schreff et al., 1998; Martin-Schild et al., 1999).

1.7. Pharmacology of Endomorphins

As concerns the pharmacological profile of endomorphins, results of numerous *in vitro* binding studies clearly demonstrated that endomorphins preferentially label the μ-opioid binding site (Hackler et al., 1997; Zadina et al., 1997; Alt et al., 1998; Goldberg et al., 1998; Hosohata et al., 1998; Harrison et al., 1999; Sugimoto-Watanabe et al., 1999; Monory et al., 2000). Endomorphin-1 and endomorphin-2 were demonstrated to have similar affinities, but greater selectivities than the μ-receptor-selective full agonist DAMGO or morphine, and displaced the general antagonist naloxone, DAMGO and other μ-selective ligands from the binding sites in a competitive and concentration-dependent manner (Horvath, 2000). Both peptides were also shown to be slightly active at the δ- and κ-opioid receptors. It has been postulated that the endomorphins act by stimulating functionally diverse subtypes of μ-opioid receptors, the putative μ_1- and μ_2-subtypes, which are presumed to be responsible for their distinct pharmacological activities (Sakurada et al., 1999, 2000; Tseng et al., 2000; Wu et al., 2002; Garzon et al., 2004). The putative μ_1-opioid receptor antagonist naloxazine has been shown to inhibit the antinociception induced by the *i.c.v.* administration of endomorphin-2 more effectively than that of endomorphin-1, whereas β-funaltrexamine inhibits both. These results suggest that the hypothetical μ_2-opioid receptors can be stimulated by both endomorphins, but the μ_1-opioid receptors only by endomorphin-2 (Wu et al., 2002; Garzon et al., 2004). It has been proposed that the μ_1-opioid receptors mediate supraspinal analgesia, while the μ_2-receptors relate to spinal analgesia and respiratory depression (Pasternak et al., 1993). However, μ-opioid receptor subtype genes have not been identified to date. At present, the receptor subtypes are suggested to result from the oligomerization of opioid receptors or unique opioid receptors, which might have undergone distinct post-translational modifications (Gomes et al., 2004; Snook et al., 2006).

1.8. Functionality of Endomorphins

As regards functionality and signal transduction, the endomorphins are mostly considered to be partial agonists of the μ-opioid receptors that are coupled to the inhibitory G_i/G_o class of G-proteins (Chen et al., 1993; Mizoguchi et al., 2001; Rónai et al., 2006). However, there are some reports in which they were classified as full agonists (Zadina et al., 1997; Hackler et al., 1997; Spetea et al., 1998). The binding of endomorphins to the μ-receptors leads to the activation of G-proteins and entails a multitude of cellular changes, including the replacement of GDP by GTP of the G_α-subunit, the opening/closure of ion channels, the reduction of cAMP formation and the stimulation of PLC/PKC system (North, 1989; Porzig, 1990; Childers, 1991; Harrison et al., 1998; Galeotti et al., 2006). In general, the efficacy of endomorphin-stimulated [^{35}S]GTPγS binding was found to be much lower than that of DAMGO (Alt et al., 1998; Harrison et al., 1998; Sim et al., 1998), whereas the efficacy of the endomorphins was similar to that of morphine (Bohn et al., 1998; Burford et al., 1998; Hosohata & Burkey, 1998). Endomorphin-1 was capable of partially antagonizing DAMGO-stimulated [^{35}S]GTPγS binding, which further confirms the partial agonist property of endomorphin-1 (Sim et al., 1998). The ED_{50} values of the endomorphins and DAMGO were consistent, indicating their similar potencies (Alt et al., 1998; Harrison et al, 1998; Hosohata & Burkey, 1998). The effects of endomorphin-1 and endomorphin-2 were comparable, and only a slight difference could be observed in terms of efficacy and potency on the use of different cell lines (SH-SY5Y human neuroblastoma, B82 fibroblast and C6 glioma cell lines) or rat and mouse brains/spinal cords (Horvath, 2000). In all studies, the effects of the endomorphins were found to be reversible by μ-opioid antagonists (Kakizawa et al., 1998; Narita et al., 1998, 1999; Spetea et al. 1998; Mizoguchi et al., 1999). Either δ- or κ-antagonists had no significant influence on the effects of peptides in stimulating [^{35}S]GTPγS binding (Kakizawa et al., 1998; Narita et al., 1998).

1.9. Tolerance and Dependence

As revealed by several studies, the chronic use of μ-opioid receptor ligands may result in the development of tolerance and/or dependence that limit their clinical

applications in pain management (Stone et al., 1997; Higashida et al., 1998; Horvath et al., 1999; Schiller et al., 1999; Shen et al., 2000; Spreekmeester & Rochford, 2000). Much effort has been devoted to delineating the μ-opioid receptor-mediated intracellular signaling mechanisms and the long-lasting molecular and cellular adaptations after acute and chronic treatments with μ-opioid receptor ligands (Burford et al., 1998; McConalogue et al., 1999; Defer et al., 2000; Heyne et al., 2000; Law et al., 2000; Nevo et al., 2000). *In vitro* and *in vivo* investigations have shown that the endomorphins induce the development of tolerance following either acute (in a high single dose) or chronic administration (Stone et al., 1997; Higashida et al, 1998; McConalogue et al., 1999; Horvath et al., 1999; Nevo et al., 2000; Hung et al., 2002; Labuz et al., 2002; Wu et al., 2001, 2003). Interestingly, the endomorphins induced the development of tolerance much faster than morphine, though endomorphin-1 required a longer pretreatment than endomorphin-2 before acute tolerance appeared (Stone et al., 1997). Additionally, cross-tolerance was observed between endomorphin-1 and morphine, but not when endomorphin-2 and morphine were co-administered (Labuz et al., 2002). The involvement of the μ-receptor subtypes (μ_1- and μ_2-) was assumed to be responsible for the diverse pharmacological effects. The endomorphins initiate the endocytosis and trafficking of the μ-opioid receptors, leading to receptor desensitization and down-regulation, similarly to DAMGO in human and rat kidney cells, stably expressing the μ-opioid receptors in a naloxone reversible manner (Burford et al., 1998; McConalogue et al., 1999). It is well known that chronic administration of μ-opioid ligands usually results in physical dependence and drug addiction. In naloxone-precipitated withdrawal experiments, the endomorphins were shown to induce physical dependence (Chen et al., 2003). Surprisingly, the severity of the symptoms resembled of those induced by the same dose of morphine. In this context, interactions are thought to exist between the opioid and the dopaminergic, NMDA and the GABAergic systems, which may co-regulate the development and outcome of the opioid-induced dependence (El-Kadi & Sharif, 1998; Sayin et al., 1998; Popik et al., 1998; Samini et al., 2000; Tokuyama et al., 2000).

2. Aims and Scope

Our research group has been involved in the development of Dmt and β-MePhe-containing endomorphin analogs for years. The application of Dmt and β-MePhe for endomorphins permitted study of the conformational requirements of opioid binding, since the introduction of these unnatural amino acids yielded several conformationally constrained analogs with diverse pharmacological potencies (Przewlocki et al., 1999; Tömböly et al., 2004; Tóth et al., 2004). Taking into account the importance of the conformation and the pharmacophore elements of the endomorphins, structure-activity studies utilizing alicyclic β-amino acids have been performed (Tóth et al., 2004; Keresztes et al., 2006). Numerous reports have been published concerning the chemistry and chemical utilization of alicyclic β-amino acids, and particularly of 2-aminocyclopentanecarboxylic acid (ACPC) and 2-aminocyclohexanecarboxylic acid (ACHC) (Fülöp, 2001; Steer et al., 2002). However, relatively few biochemical and pharmacological data have been published so far on the effects of ACPC/ACHC substitutions in opioid peptides (Mierke et al., 1990; Yamazaki et al., 1991; Bozó et al., 1997). The five- and six-membered, saturated-unsaturated alicyclic β-amino acids proved to be attractive targets for endomorphin engineering as a consequence of their unique structural features. All alicyclic β-amino acids have two chiral centers, which extend the number of isomers, and the incorporation of these amino acids furnishes these bioactive peptides with high proteolytic stability (Fülöp 2001; Tóth et al., 2004). Taking these facts into consideration, our aims were:

- To synthetize Dmt and/or ACPC/ACHC or ΔACPC/ΔACHC-containing endomorphins in order to obtain proteolitically stable analogs.
- To test the synthetized analogs in receptor-binding studies in order to study the effects of the conformational modifications on the affinity and selectivity.
- To study the agonist/antagonist features of the newly obtained analogs, selected on the basis of the results of preliminary receptor-binding assays (displacement), using the ligand-stimulated [^{35}S]GTPγS functional assay.

- To radiolabel one of the most promising analogs on the basis of the results of receptor-binding characterizations in order to obtain a proteolitically stable radiolabeled μ-opioid receptor agonist.
- To fully characterize the new radioligand in *in vitro* receptor-binding experiments.

3. Materials and Methods

3.1. Chemicals

The saturated and unsaturated alicyclic β-amino acids were synthetized, purified and kindly provided by Dr. Ferenc Fülöp (Department of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary). All the opioid peptides containing ACPC/ACHC or their unsaturated forms, and also endomorphin-1, endomorphin-2 and Ile^{5,6}-deltorphin-2, were synthetized in the Isotope Laboratory by using solid-phase peptide synthesis protocols, as published by Tömböly et al. (2004). DAMGO and H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE) were purchased from Sigma-Aldrich Kft. (Budapest, Hungary). [^{3}H]DAMGO (1.6 TBq mmol^{-1}, 43 Ci mmol^{-1}), [^{3}H]Ile^{5,6}-deltorphin-2 (0.8 TBq mmol^{-1}, 21 Ci mmol^{-1}), [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 (1.4 TBq mmol^{-1}, 38 Ci mmol^{-1}) and [^{3}H][(*1S,2R*)ACHC]^{2}endomorphin-2 (2.4 TBq mmol^{-1}, 63.5 Ci mmol^{-1}) were prepared in our laboratory from the appropriate unsaturated or halogenated peptide derivatives by tritium saturation or dehalogenation, as published by Tóth et al. (Neuropeptide Protocoll, Vol. 73, p 219, 1997). Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) was purchased from Amersham (GE Healthcarem, United Kingdom). Morphine hydrochloride, naloxone hydrochloride (Nx), norbinaltorphimine (NorBNI) and naltrindole (NTI) were synthetized and kindly provided by Dr. Sándor Hosztafi (Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary). *Trans*(±)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U-50,488) was obtained from Upjohn Co. (Kalamazoo, MI, USA). GF/B and GF/C filters were purchased from Whatman International Ltd. (Maidstone England, England). Naloxone methiodide, Tris-(hydroxymethyl)-aminomethane (TRIS, free base), Bradford reagent, bovine serum

albumin (BSA), sucrose, guanosine 5'-[β,γ-imido]-triphosphate trisodium salt (Gpp(NH)p), hydrochloric acid (HCl 37%) and anisole were purchased from Sigma-Aldrich Kft. (Budapest, Hungary). *N,N*-dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF), trifluoroacetic acid (TFA), dichloromethane (DCM), diisopropylethylamine (DIEA), *N*-hydroxybenzotriazole (HOBt), ninhydrin, acetonitrile (ACN), $Pd/BaSO_4$ catalyst and triethylamine (TEA) were delivered by Merk Kft. (Budapest, Hungary). Acetic acid (AcOH) and ethanol (EtOH) were from Molar Kft. (Budapest, Hungary), while diethylether, 2,5-diphenyloxazole (PPO) and 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene (POPOP) were from Reanal (Budapest, Hungary). All the solvents were of analytical grade. The dimethyl sulfoxide (DMSO-d_6) used for the ^{1}H-NMR measurements was purchased from Cambridge Isotopes. Tritium gas was obtained from Techsnabexport (Moscow, Russia), 4-methylbenzhydrylamine resin (MBHA) from Bachem AG (Bubendorf, Switzerland), and hydrogen fluoride from PRAXAIR N.V. (Oevel, Belgium).

3.2. Analytical Methods

TLC (Thin Layer Chromatography) was performed on chiral TLC plates (Macherey Nagel, Dürer, Germany) or on silica gel 60 F_{254} (Merck, Darmstadt, Germany), using the following solvent systems: (A) 1-butanol/acetic acid/water (4:1:1), (B) acetonitrile/methanol/water (4:1:1). Spots were visualized under UV light or with ninhydrin reagent. HPLC (High Performance Liquid Chromatography) separation and analysis of all compounds were performed using an HPLC system consisting of a Merck (Merck KGaA, Darmstadt, Germany) L-7100 pump, a Shimadzu (Shimadzu Co., Kyoto, Japan) SIL-6B autosampler, a Shimadzu SCL-6B system controller and a Merck L-7400 UV-VIS detector, operating at 215 nm with a Hitachi D-7000 HPLC system manager. For radioligands, a Jasco PU-980 Intelligent HPLC pump, a Jasco LG-980-02 ternary gradient unit, a Jasco UV-975 Intelligent UV/VIS detector (Jasco International Co., Tokyo, Japan) and a Packard A-500 radiochromatography detector were applied (Packard BioScience Co., Meriden, CT, USA) with an Ultima-flo M liquid scintillation cocktail. The samples were analyzed on Vydac 218TP1010 (250×10 mm, 12 μm) and Vydac

218TP54 (250×4.6 mm, 5 μm) C_{18} reversed-phase columns at a flow rate of 4 ml $min.^{-1}$ and 1 ml $min.^{-1}$, respectively, at room temperature. The mobile phase was composed of 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile, and gradient elution was carried out from 15% up to 40% of organic modifier. The eluents were sonicated before use. Injected samples were filtered through a 0.45 μm Whatman PTFE syringe filter (Inc, Clifton, USA). The purities of the inactive peptides were confirmed by mass spectrometry, using a Reflex III MALDI-TOF instrument (Bruker Bremen, Germany) in the positive reflectron mode. For binding experiments, incubation mixtures were collected by a M24R Brandel Cell Harvester (Gaithersburg, MD, USA). Filter activities were measured in a TRI-CARB 2100TR Liquid Scintillation Analyzer (Packard) and recorded simultaneously by printer and disk.

3.3. Peptide Synthesis, Purification and Determination of Configurations of the Incorporated Alicyclic β-Amino Acids

The peptide analogs were synthetized by using *N*-α-*t*-Boc-protected amino acids and MBHA resin to obtain C-terminal amides (Tömböly et al., 2004). All amino acids were protected manually with di-*tert*-butyl-dicarbonate. *Cis*-(*1S,2R/1R,2S*)ΔACPC/ACHC, c*is*-(*1S,2R/1R,2S*)ACPC, *cis*-(*1S,2R/1R,2S*)ACHC, *trans*-(*1S,2S/1R,2R*)ACPC and *trans*-(*1S,2S/1R,2R*)ACHC were incorporated in racemic form into the endomorphins. The Boc-L-Dmt was HPLC pure. The coupling reagents were *N*-hydroxybenzotriazole and *N,N'*-dicyclohexylcarbodiimide used for peptide elongation. Each coupling step was monitored by the Kaiser test. The protecting groups were removed by washing the resin with a combination of 50% TFA and 48% DCM containing 2% anisole. The resin was washed repeatedly with EtOH and DCM between two coupling steps. Neutralization was carried out with 20% DIEA, followed by repeated washing with DCM. The peptides were cleaved with anhydrous liquid HF (5 ml $(g\ resin)^{-1}$, 1 h, 0 ^{o}C) in the presence of anisole (1 ml $(g\ resin)^{-1}$). After the evaporation of the HF, diethylether was used to wash the resin and peptides. The peptides were eluted with concentrate AcOH, and the solution was then diluted with water and lyophilized. The yield of the crude peptides varied in the interval 60-80%, depending on the unnatural β-

amino acid incorporated. The diastereomers were separated by using semipreparative RP-HPLC and a Vydac 218TP1010 column with a linear gradient of 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile. The yield of the pure peptides ranged from 20% up to 80%, depending on the diastereomers. Purities were found to be over 95%, as assessed by analytical RP-HPLC. The molecular weights of the analogs were confirmed by mass spectrometry. The absolute configurations of the incorporated β-amino acids were determined after acidic hydrolysis of the diastereomer peptides (6 M HCl, 24 h, 110 ^{o}C) (Péter et al., 1995). The acidic hydrolyzates were separated by analytical RP-HPLC to isolate the appropriate β-amino acids. The absolute configurations of the purified alicyclic β-amino acids were determined by using chiral TLC (1-butanol/acetic acid/water (4:1:1), acetonitrile/methanol/water (4:1:1)) and the R_f values, which were then compared with those of optically pure alicyclic β-amino acid standards. GITC derivatization of the amino acid mixtures, followed by analytical HPLC, was used to confirm the configurations of the alicyclic β-amino acids (Péter et al., 1995). The retention times of the derivatized alicyclic β-amino acids were compared with those of the derivatized β-amino acid standards.

3.4. Radiolabeling of [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 and [^{3}H][(*1S,2R*)ACHC]^{2}endomorphin-2

The precursor peptides for the radiolabeling of ([(*1S,2R*)ΔACPC]^{2}endomorphin-2 and [(*1S,2R*)ΔACHC]^{2}endomorphin-2) were synthetized and purified manually according to the procedure described above. The precursors (2 mg each) were dissolved in DMF, and 10 mg Pd/$BaSO_4$ was added as catalyst in the presence of 1μl TEA. The tritium gas was liberated prior to the reaction by heating uranium tritide at 300-400 ^{o}C, using our home-made apparatus. The mixtures were stirred for 1-2 hours in the presence of $^{3}H_2$ at room temperature. At the end of the saturation reaction, the catalyst was removed by filtration through a Whatman GF/C glass fiber filter. The filtrates were evaporated, and then washed several times with a 1:1 EtOH:water mixture to remove labile tritium. The total activities of the crude radioligands were found to be 193 mCi (7.1 GBq) for [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 and 171 mCi (6.3 GBq) for

[^{3}H][(*1S,2R*)ACHC]^{2}endomorphin-2. The radioligands were purified by RP-HPLC under the conditions described above. The specific activities were determined via calibration curves prepared with the appropriate inactive saturated standards, and were found to be 38 Ci mmol^{-1} (1.4 TBq mmol^{-1}) for [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 and 63.5 Ci mmol^{-1} (2.4 TBq mmol^{-1}) for [^{3}H][(*1S,2R*)ACHC]^{2}endomorphin-2.

3.5. Rat Brain Membrane Preparation

Rats (male, Wistar, 250-300 g body weight) were kept in groups of four under 12:12-h light/dark cycle, with free access to standard food and water, until the time of sacrifice. Animals were handled according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII. tv. Section 32). A crude membrane preparation was prepared from the whole brain of rats, without the cerebellum and hippocampus (Bozó et al., 1997). The brains were removed and homogenized in a 30-fold excess of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a teflon-glass Braun homogenizer at 1500 rpm. The homogenate was centrifuged at 20,000 x g for 25 min. and the resulting pellet was resuspended in the same volume of cold buffer, followed by incubation at 37 ^{o}C for 30 min. to remove endogenous ligands. Centrifugation was then repeated and the final pellet was taken up in 5-fold amount of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored in liquid nitrogen. Before the experiments, brains were thawed, diluted with the working buffer for either radioligand or [^{35}S]GTPγS binding assays, and centrifuged at 20,000 x g to remove sucrose. The pellet was homogenized with a Dounce in the appropriate volume of buffer. Protein content was determined by the method of Bradford, using BSA as a standard (Bradford, 1976).

3.6. Radioligand Binding Assay

In competitive binding experiments**,** assay conditions were optimized and varied, depending upon the radioligand applied. The following conditions were set to assess inhibitory constants: [^{3}H]DAMGO (25 ^{o}C, 1 h, GF/C filter, glass tube), [^{3}H]Ile^{5,6}deltorphin-2 (35 ^{o}C, 45 min., GF/B filter, plastic tube, 0.25 mg BSA/tube). The

incubation mixtures were made up to a final volume of 1 ml with 50 mM Tris-HCl buffer (pH 7.4) and samples were incubated in a shaking water bath at the appropriate temperature. For binding experiments with [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2, experimental tools (tips and tubes) were silanized to prevent adsorption, and each incubation was performed at 25 ^{o}C. The time course of association was measured by incubating 0.4 nM radioligand with a protein concentration described below. In the dissociation experiments, the radioligand was incubated with the protein until equilibrium, followed by the addition of 10 μM naloxone to initiate dissociation. Saturation binding experiments were performed with increasing concentrations (0.1-16 nM) of [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2. The protein concentration was varied between 0.3 and 0.5 mg/test tube. Competition binding experiments were performed by incubating the membranes with 0.5 nM [^{3}H]DAMGO, 2 nM [^{3}H]Ile^{5,6}deltorphin-2 or 0.4 nM [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2, and increasing concentrations (10^{-12}-10^{-5} M) of unlabeled endomorphin analogs. Nonspecific binding was determined with 10 μM naloxone and subtracted from the total binding to yield the specific binding. Incubation was initiated by the addition of the membrane suspension and stopped by rapid filtration over Whatman GF/C or GF/B glass fiber filters, using a Brandel Cell Harvester. Filters were washed with 3 x 5 ml of ice-cold Tris-HCl buffer (pH: 7.4), and then dried at 37 ^{o}C for 2-3 h and the radioactivity was measured in a toluene-based scintillation cocktail, using a TRI-CARB 2100TR Liquid Scintillation Counter. The inhibitory constants, K_i (a logarithmic concentration of a drug that gives 50% of the inhibition of the radioligand binding), were calculated from the displacement curves by using non-linear least-square curve fitting and the Cheng-Prusoff equation (Cheng et al., 1973). Kinetic data were calculated to obtain the rate constant for association (k_a) and dissociation (k_d). Nonlinear regression analysis of the direct saturation isotherms was performed to yield the equilibrium dissociation constant (K_d) and receptor density (B_{max} – the number of maximal binding sites). Each experiment was performed in duplicate and analyzed by the one/two-site binding competition fitting option of the GraphPad Prism Software, and are expressed as means ± S.E.M of at least three independent experiments, each carried out in duplicate.

3.7. Ligand-stimulated [^{35}S]GTPγS Binding Assay

Rat brain membranes (15 μg protein/tube) were incubated with 0.05 nM [^{35}S]GTPγS and 10^{-10}-10^{-5} M opioids in the presence of 30 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 1 mM EGTA in 50 mM Tris-HCl pH 7.4 buffer for 60 min. at 30 ^{o}C (Bozó et al., 2000). Basal binding was measured in the absence of opioids and was corrected for the nonspecific binding to yield the specific binding. Nonspecific binding was determined with 10 μM unlabeled GTPγS. The reaction was initiated by the addition of the protein and terminated by the addition of 5 ml ice-cold buffer (50 mM Tris-HCl, pH: 7.4) to the vials and filtering the samples through a Whatman GF/B glass fiber filter with a Brandel Cell Harvester. Vials were washed three times with 5 ml of ice-cold buffer, and then dried at 37 ^{o}C for 3 h. The filter-bound activity was measured in a toluene-based cocktail, using a TRI-CARB 2100TR Liquid Scintillation Counter. Ligand stimulations were expressed as percentage of the specific [^{35}S]GTPγS binding over the basal activity. For [^{35}S]GTPγS experiments, EC_{50} (the effective concentration, the concentration of a drug that gives 50% of the maximal response) and E_{max} (the maximal efficacy, the maximal stimulation of a drug over the basal activity) were determined by using the sigmoid dose-response curve-fitting option of the GraphPad Prism Software (version 4.0, San Diego, CA, USA). The results are means ± S.E.M of at least three independent experiments.

4. Results

4.1. Synthesis of Conformationally Constrained Endomorphin Analogs

New endomorphin analogs were synthetized by solid-phase peptide synthesis using L-Dmt, racemic saturated or unsaturated alicyclic β-amino acids or their combinations to obtain potent, conformationally constrained, proteolitically stable endomorphin derivatives. The analytical properties of the twenty-four analogs are reported in **Table 3**. The chirally pure L-Dmt was obtained from racemic *N*-trifluoroacetyl-Dmt by enzymatic treatment with carboxypeptidase A (Tóth et al., 2004). Racemic saturated and unsaturated *cis*-ACPC and *cis*-ACHC were prepared from the corresponding cycloalkene by chlorosulfonyl isocyanate addition, followed by ring opening procedure with hydrochloric acid. The *trans* counterparts were prepared from the corresponding 1,2-dicarboxylic anhydrides after amidation, followed by Hofmann degradation with hypobromite. The enantiomers of *cis*-ACPC and *cis*-ACHC were prepared by lipolase-catalyzed enantioselective ring opening of the corresponding β-lactams, while the *trans*-ACPC and *trans*-ACHC enantiomers were obtained by enzymatic hydrolysis of alicyclic β-amino acid esters (Fülöp, 2001). The absolute configurations of the incorporated β-amino acids were determined after acidic hydrolysis of the diastereomer peptides (6 M HCl, 24 h, 110 ^{o}C) or after catalytic hydrogenation of the unsaturated diastereomers. The retention times of the catalytically saturated peptides were compared with those of peptide standards. For the former procedure, acidic hydrolyzates were separated by analytical RP-HPLC and the absolute configurations of the purified alicyclic β-amino acids were determined by using chiral TLC and the R_f values, which were then compared with those of optical pure alicyclic β-amino acid standards. GITC derivatization of the amino acid mixtures, followed by analytical HPLC, was used to confirm the configurations of the alicyclic β-amino acids. The retention times of the derivatized alicyclic β-amino acids were compared with those of the derivatized β-amino acid standards (Péter et al., 1995).

Table 3. Analytical data of the native peptides and their synthetized analogs.

No.	Peptide	Formula	TLC[a] R_f (A)	R_f (B)	HPLC [b] (k')	MS $[M]_{cal.}$	MS $[M + H]^+$
1	endomorphin-1	$C_{34}H_{38}N_6O_5$	0.55	0.45	3.29	610	611.3
2	[(*1S,2R*)ACPC]^{2}endomorphin-1	$C_{35}H_{41}N_6O_5$	0.62	0.30	4.69	624	625.3
3	[(*1R,2S*)ACPC]^{2}endomorphin-1	$C_{35}H_{41}N_6O_5$	0.56	0.45	3.95	624	625.3
4	[(*1S,2S*)ACPC]^{2}endomorphin-1	$C_{35}H_{41}N_6O_5$	0.59	0.45	4.44	624	625.3
5	[(*1R,2R*)ACPC]^{2}endomorphin-1	$C_{35}H_{41}N_6O_5$	0.55	0.46	4.50	624	625.3
6	[(*1S,2R*)ACHC]^{2}endomorphin-1	$C_{36}H_{43}N_6O_5$	0.63	0.32	2.92	638	639.3
7	[(*1R,2S*)ACHC]^{2}endomorphin-1	$C_{36}H_{43}N_6O_5$	0.57	0.42	2.79	638	639.3
8	[(*1S,2S*)ACHC]^{2}endomorphin-1	$C_{36}H_{43}N_6O_5$	0.62	0.45	4.40	638	639.3
9	[(*1R,2R*)ACHC]^{2}endomorphin-1	$C_{36}H_{43}N_6O_5$	0.54	0.43	4.47	638	639.3
10	endomorphin-2	$C_{32}H_{37}N_5O_5$	0.55	0.42	3.09	571	572.3
11	[(*1S,2R*)ΔACPC]^{2}endomorphin-2	$C_{33}H_{38}N_6O_5$	0.88	0.29	4.68	583	584.3
12	[(*1S,2R*)ΔACHC]^{2}endomorphin-2	$C_{34}H_{40}N_6O_5$	0.75	0.31	4.95	597	598.3
13	[(*1S,2R*)ACPC]^{2}endomorphin-2	$C_{33}H_{40}N_6O_5$	0.63	0.30	4.72	585	586.3
14	[(*1R,2S*)ACPC]^{2}endomorphin-2	$C_{33}H_{40}N_6O_5$	0.53	0.42	3.84	585	586.3
15	[(*1S,2S*)ACPC]^{2}endomorphin-2	$C_{33}H_{40}N_6O_5$	0.59	0.44	4.12	585	586.3
16	[(*1R,2R*)ACPC]^{2}endomorphin-2	$C_{33}H_{40}N_6O_5$	0.55	0.45	4.37	585	586.3
17	[(*1S,2R*)ACHC]^{2}endomorphin-2	$C_{34}H_{42}N_6O_5$	0.63	0.30	4.89	599	600.3
18	[(*1R,2S*)ACHC]^{2}endomorphin-2	$C_{34}H_{42}N_6O_5$	0.56	0.40	4.11	599	600.3
19	[(*1S,2S*)ACHC]^{2}endomorphin-2	$C_{34}H_{42}N_6O_5$	0.62	0.38	4.01	599	600.3
20	[(*1R,2R*)ACHC]^{2}endomorphin-2	$C_{34}H_{42}N_6O_5$	0.58	0.42	4.57	599	600.3
21	[Dmt]^1[(*1S,2R*)ACPC]^{2}endomorphin-2	$C_{35}H_{44}N_6O_5$	0.81	0.75	5.29	613	614.3
22	[Dmt]^1[(*1R,2S*)ACPC]^{2}endomorphin-2	$C_{35}H_{44}N_6O_5$	0.73	0.71	4.17	613	614.3
23	[Dmt]^1[(*1S,2R*)ACHC]^{2}endomorphin-2	$C_{36}H_{46}N_6O_5$	0.80	0.74	5.59	627	628.3
24	[Dmt]^1[(*1R,2S*)ACHC]^{2}endomorphin-2	$C_{36}H_{46}N_6O_5$	0.76	0.73	4.38	627	628

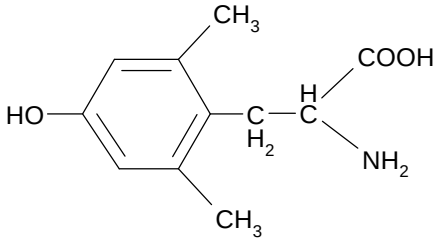
[a] Retention factors on silica gel 60 F_{254} plates. Solvent systems: (A) 1-butanol/acetic acid/water (4:1:1), (B) acetonitrile/methanol/water (4:1:1).[b] Capacity factors for the Vydac 218TP54 (25 × 0.46 cm, d_p = 5 μm) column. The gradient was from 15% up to 40% ACN during 25 min., at a flow rate of 1 ml $min.^{-1}$, λ = 215 nm.

The solid-phase peptide synthesis gave relatively high yields of the crude peptide analogs (60-80%), with a low percentage of contamination. The alicyclic β-amino acids and the double-substituted Dmt^1- and alicyclic β-amino acid-containing peptides were purified by preparative RP-HPLC. The purities of the peptides were determined by using analytical RP-HPLC and TLC chromatography systems. The ratio of the diastereomeric peptides after the synthesis was nearly 3:1 with respect to (*1R,2S/1S,2S*)ACPC/ACHC and (*1S,2R/1R,2R*)ACPC/ACHC-containing analogs. A similar tendency of the diastereomeric peptide ratio was observed for the Dmt/ACPC/ACHC and unsaturated ACPC/ACHC-containing analogs. All the new endomorphin analogs were white, water-soluble powders. Representative chemical structures of the incorporated amino acid residues are presented in **Figure 2**.

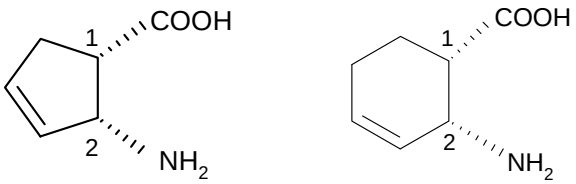
4.2. Radiolabeling of [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 and [^{3}H][(*1S,2R*)ACHC]^{2}endomorphin-2

On the basis of the results of radioligand receptor-binding experiments, two potent endomorphin analogs with unsaturated alicyclic β-amino acids were synthetized and purified to produce radiolabeled, proteolitically stable endomorphin analogs. The double bond in the amino acid ring served as a potential site for catalytic saturation with tritium, which finally yielded the corresponding tritiated compounds. The radiolabeling of [(*1S,2R*)ΔACPC]^{2}endomorphin-2 and [(*1S,2R*)ΔACHC]^{2}endomorphin-2 analogs was carried out with tritium gas and $Pd/BaSO_4$ catalyst. The saturation reaction provided the appropriate radiolabeled analogs with total radioactivities of 193 mCi (7.1 GBq) and 171 mCi (6.3 GBq) for the crude [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 and [^{3}H][(*1S,2R*)ACHC]^{2}endomorphin-2, respectively. The specific activities of the purified radioligands were measured to be 38 Ci mmol^{-1} (1.4 TBq mmol^{-1}) and 63.5 Ci mmol^{-1} (2.4 TBq mmol^{-1}) for [^{3}H][(*1S,2R*)ACPC^2]endomorphin-2 and [^{3}H][(*1S,2R*)ACHC^2]endomorphin-2, respectively. The radioligands were purified by RP-HPLC and stored at a concentration of 1 mCi ml^{-1} in liquid nitrogen until further operation.

2,6-dimethyltyrosine (Dmt)

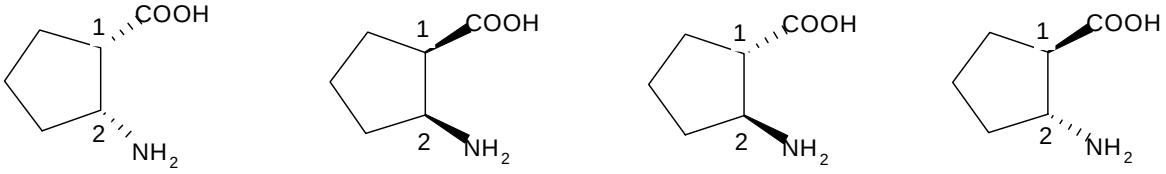


2-aminocyclopentene/hexenecarboxylic acid (ΔACPC/ΔACHC)



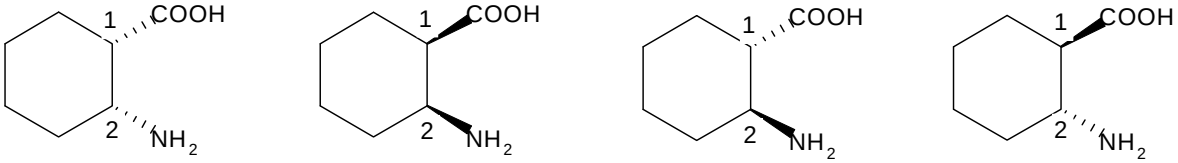
cis-(1S,2R)- *cis-(1S,2R)-*

2-aminocyclopentanecarboxylic acid (ACPC)



cis-(1S,2R)- *cis-(1R,2S)-* *trans-(1S,2S)-* *trans-(1R,2R)-*

2-aminocyclohexanecarboxylic acid (ACHC)



cis-(1S,2R)- *cis-(1R,2S)-* *trans-(1S,2S)-* *trans-(1R,2R)-*

Figure 2. Representative chemical structures of the incorporated unnatural amino acids. The application of the alicyclic β-amino acid ACPC and ACHC resulted in diastereomer pairs due to the two chiral centers, while Dmt provided only one corresponding analog.

4.3. Radioligand Binding of Alicyclic β-Amino Acid-Containing Endomorphin Analogs

The pharmacological properties of the newly synthetized analogs were determined in radioligand-binding experiments. [^{3}H]DAMGO, a well-studied, highly-selective μ-receptor radioligand, was used to test the affinity of the analogs for the μ-opioid receptors. The widely-known, synthetic δ-receptor peptide derivative the [^{3}H]Ile^{5,6}deltorphin-2 was applied to measure δ-receptor affinities and selectivities. The inhibitory constants (K_i nM) and selectivities (K_i^{δ}/K_i^{μ}) of the eighteen analogs are presented in **Table 4**. Endomorphin-1 and endomorphin-2 were also characterized for comparison and the values were consistent with literature data (Zadina et al., 1997; Spetea et al., 1998; Tóth et al., 2004). All the new endomorphin derivatives displaced the radiolabelled μ- and δ-receptor specific opioid ligands from a single binding site in a concentration-dependent manner. In general, Pro^2 substitution to *cis*-(*1S,2R*)ACPC/ACHC^2 resulted in the most potent endomorphin analogs, with nanomolar inhibitory constants measured for [^{3}H]DAMGO. The rank order of potency of the monosubstituted derivatives was determined to be as follows: compounds **1 > 10 > 6 > 13 = 17 > 2 > 11 > 12 > 16 > 5 > 20 > 4 > 19 > 3 > 15 > 7 > 8 > 9 > 18 >> 14**. The *cis*-(*1R,2S*)ACPC/ACHC^2-containing counterparts exhibited much higher inhibitory values as compared with those detailed above. Replacement with either the *trans*-(*1S,2S*)ACPC^2 or *trans*-(*1S,2S*)ACHC^2 amino acid surrogates yielded analogs (compounds **4**, **8**, **15** and **19**) with very weak potencies. Incorporation of the *trans*-(*1R,2R*)ACPC^2 or *trans*-(*1R,2R*)ACHC^2 alicyclic β-amino acids into the endomorphins resulted in relatively potent derivatives (compounds **5**, **16** and **20**), except in the case of compound **9**, which exhibited a somewhat higher inhibitory constant, similarly to the *trans*-(*1S,2S*)ACPC/ACHC^2-containing analogs. As regards these differences, it seems likely that the chirality is a more important feature in effecting the binding potency than the ring size of the alicyclic β-amino acids. As regards these differences, it seems likely that the chirality is a more important feature in effecting the binding potency than the ring size of the alicyclic β-amino acids.

Table 4. Inhibitory constants (K_i) of endomorphin analogs containing alicyclic β-amino acids, measured in rat brain membrane preparations.

No.	Peptide	Inhibitory constant		Selectivity
		K_i^{μ} (nM)[a]	K_i^{δ} (nM)[b]	K_i^{δ}/K_i^{μ}
1	endomorphin-1	0.74 ± 0.03	1,909 ± 233	2,580
2	[(*1S,2R*)ACPC]^{2}endomorphin-1	3.6 ± 0.3	1,341 ± 122	373
3	[(*1R,2S*)ACPC]^{2}endomorphin-1	275 ± 41	> 10,000	-
4	[(*1S,2S*)ACPC]^{2}endomorphin-1	86 ± 8	3,075 ± 128	36
5	[(*1R,2R*)ACPC]^{2}endomorphin-1	16 ± 2	670 ± 31	42
6	[(*1S,2R*)ACHC]^{2}endomorphin-1	1.8 ± 0.5	2,763 ± 213	1,535
7	[(*1R,2S*)ACHC]^{2}endomorphin-1	741 ± 61	> 10,000	-
8	[(*1S,2S*)ACHC]^{2}endomorphin-1	883 ± 100	9,617 ± 6,262	11
9	[(*1R,2R*)ACHC]^{2}endomorphin-1	1,033 ± 81	> 10,000	-
10	endomorphin-2	1.3 ± 0.2	5,652 ± 202	4,348
11	[(*1S,2R*)ΔACPC]^{2}endomorphin-2	5.8 ± 0.6	4,947 ± 840	853
12	[(*1S,2R*)ΔACHC]^{2}endomorphin-2	7.6 ± 0.6	591 ± 89	78
13	[(*1S,2R*)ACPC]^{2}endomorphin-2	2.4 ± 0.1	4,798 ± 310	1,999
14	[(*1R,2S*)ACPC]^{2}endomorphin-2	4,435 ± 645	> 10,000	-
15	[(*1S,2S*)ACPC]^{2}endomorphin-2	374 ± 61	4,228 ± 210	11
16	[(*1R,2R*)ACPC]^{2}endomorphin-2	14 ± 3	1,411 ± 275	100
17	[(*1S,2R*)ACHC]^{2}endomorphin-2	2.4 ± 0.1	812 ± 18	338
18	[(*1R,2S*)ACHC]^{2}endomorphin-2	1,984 ± 194	> 10,000	-
19	[(*1S,2S*)ACHC]^{2}endomorphin-2	166 ± 40	> 10,000	-
20	[(*1R,2R*)ACHC]^{2}endomorphin-2	21 ± 1	439 ± 41	21

[a][^{3}H]DAMGO (K_d = 0.5 nM) and [b][^{3}H]Ile^{5,6}deltorphin-2 (K_d = 2.0 nM) were used as radioligands for μ- and δ-opioid receptors. Nonspecific binding was determined with 10^{-5} M naloxone. No selectivities were assigned where inhibitory constants proved to be higher than 10.000 nM. K_i values were calculated according to the Cheng–Prusoff equation: $K_i=EC_{50}/(1+[ligand]/K_d)$, where the K_d values shown were obtained from radioligand saturation experiments.

Furthermore, these data strongly suggest that the *trans*-(*1R,2R*)ACPC/ACHC[2]-containing analogs tend to adopt conformations which readily interact with the active conformational states of the receptor-binding sites.

The overall reflection of the displacement experiments, measured against the δ-specific [^{3}H]Ile^{5,6}-deltorphin-2, is that each ligand substituted by these residues binds with low affinities to the δ-receptor. The rank order of potency of the ligands tested for the [^{3}H]Ile^{5,6}-deltorphin-2 was measured to be as follows: compounds **20** > **12** > **5** > **17** > **2** ≈ **16** > **1** > **6** > **4** > **15** > **13** ≈ **11** > **10** >> **8** > **3**, **7**, **9**, **14**, **18**, **19**. The somewhat low selectivity values (K_i^{δ}/K_i^{μ}) calculated from the [^{3}H]DAMGO and [^{3}H]Ile^{5,6}-deltorphin-2 experiments clearly indicate the profound loss of selectivity. The most selective ligands to be tested in this assay were the parent endomorphins (K_i^{δ}/K_i^{μ}·= 2,580-4,348), in comparison with the ACPC/ACHC-containing analogs. There were only two ligands, compounds **6** (K_i^{δ}/K_i^{μ} = 1,535) and **13** (K_i^{δ}/K_i^{μ} = 1,999), that displayed comparable values similar to those of their parent ligands.

4.4. [^{35}S]GTPγS Functional Binding Assay of Alicyclic β-Amino Acid-Containing Endomorphin Analogs

The ligands to be tested were selected on the basis of their inhibitory constants, measured against [^{3}H]DAMGO. The potency (EC_{50}) and efficacy (E_{max}) values in stimulating [^{35}S]GTPγS binding for the tested compounds are given in **Table 5**. These values were compared with those of the prototypic full agonist DAMGO, to predict the partial/full agonist features of the new ligands. The DAMGO-stimulated [^{35}S]GTPγS binding was dose-dependent and saturable, with EC_{50} = 145 ± 47 nM, E_{max} = 170 ± 3.3%, in accordance with literature data (Alt et al., 1998; Harrison et al., 1998; Hosohata et al., 1998). Compounds **2**, **5**, **13** and **17** were shown to be the most potent analogs, producing dose-dependent increases in [^{35}S]GTPγS binding with EC_{50} values of 342 ± 81, 396 ± 70, 353 ± 54 and 569 ± 114 nM. It is noteworthy that the parent ligands yielded slightly higher potencies (lower EC_{50}) than that of DAMGO.

Table 5. Summary of [^{35}S]GTPγS functional assays carried out with the selected ACPC or ACHC-containing endomorphins in rat brain membrane preparations.

No.	Peptide	EC_{50} (nM)	E_{max} (%)	Predicted efficacy
0	DAMGO	145 ± 47	170 ± 3	Full agonist
1	endomorphin-1	104 ± 29	156 ± 4	Partial agonist
10	endomorphin-2	78 ± 16	148 ± 5	Partial agonist
2	[(*1S,2R*)ACPC]^{2}endomorphin-1	342 ± 81	157 ± 5	Partial agonist
4	[(*1S,2S*)ACPC]^{2}endomorphin-1	4,148 ± 1,575	129 ± 9	Partial agonist
5	[(*1R,2R*)ACPC]^{2}endomorphin-1	396 ± 70	137 ± 6	Partial agonist
13	[(*1S,2R*)ACPC]^{2}endomorphin-2	353 ± 54	163 ± 4	Partial agonist
16	[(*1R,2R*)ACPC]^{2}endomorphin-2	2,082 ± 773	154 ± 5	Partial agonist
17	[(*1S,2R*)ACHC]^{2}endomorphin-2	569 ± 114	181 ± 9	Full agonist
20	[(*1R,2R*)ACHC]^{2}endomorphin-2	1,834 ± 394	148 ± 1	Partial agonist

Dose-response curves of the listed peptides were measured as described in the Methods. EC_{50} and E_{max} values were calculated by GraphPad Prism. Data were expressed as % stimulation of the basal activities, i.e. the binding in the absence of peptides that was defined as 100%. Means ± S.E.M., n ≥ 3, each performed in triplicate.

Another surprising outcome of the results is that compound **5** was found to display high potency, despite carrying a *trans* residue, while the other *trans* residue-carrying analogs (compounds **4, 16** and **20**) proved to have lower potencies. The rank order of agonist potency was compounds **10** > **1** > **0** > **2** ≈ **5** ≈ **13** > **17** > **20** ≈ **16** >> **4**.

As concerns efficacy, the peptide analogs used in the study produced a wide range of values, from 129% up to 181%. Compound **17** gave the highest stimulation rate (E_{max} = 181%), similarly to DAMGO (E_{max} = 170%), and hence was classified as a full agonist. Although, it was classified to be a full agonist, its potency was far worse than that of DAMGO. Compounds **2**, **4, 5, 13**, **16** and **20** provided efficacies comparable to those of the parent endomorphins (E_{max} = 156 ± 4% and 148 ± 5%, respectively), and were predicted to be partial agonist (Sim et al., 199; Rónai et al., 2006). To test the opioid receptor specificity of the compounds, experiments were performed in the presence of 10 μM naloxone (curves not shown). This universal opioid receptor antagonist completely

blocked the ligand-stimulated effects at all agonist concentrations, thereby confirming that the synthetic derivatives retained their opioid-receptor specificities.

4.5. Receptor Binding Characterization of [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2

On the basis of ligand-activated [^{35}S]GTPγS functional assay, receptor binding and stability experiments, one ligand, compound **13,** was chosen for radiolabeling and overall receptor-binding characterization. All the binding assays were performed on rat brain membrane preparations. Association experiments were carried out with 0.4 nM [^{3}H][*(1S,2R)*ACPC]^{2}endomorphin-2 at 25 °C for 150 min. The specific binding was found to reach the steady state by 30 min. and remain stable (curve not shown). The nonspecific binding at this radioligand concentration under equilibrium conditions was about 35% of the total binding. The pseudo-first-order rate constant was 0.148 ± 0.009 min^{-1}, from which the second-order association rate constant, k_a, was calculated to be 0.079 ± 0.001 nM^{-1} x min^{-1}. Dissociation was initiated by adding 10 μM unlabeled naloxone at equilibrium, and proceeded with monophasic kinetics with a dissociation rate constant of k_d = 0.126 ± 0.015 min^{-1}. The kinetically derived equilibrium dissociation constant, K_d was calculated from these rate constants to be 1.6 ± 0.3 nM. The specific binding of [^{3}H][*(1S,2R)*ACPC]^{2}endomorphin-2 was saturable and of high affinity. Single-site binding, resulting in K_d = 1.8 ± 0.2 nM and receptor density, B_{max} = 345 ± 27 fmol x (mg protein)$^{-1}$, was calculated from the direct saturation plots. Both 100 mM Na^+ and 100 μM Gpp(NH)p decreased the affinity (K_d = 6.7 ± 0.6 nM and B_{max} = 281.8 ± 35 fmol (mg protein)$^{-1}$ for Na^+, K_d = 4.3 ± 0.4 nM and B_{max} = 232.8 ± 23 fmol (mg protein)$^{-1}$ for Gpp(NH)p), reflecting the agonist character of the radioligand, in agreement with the findings from comprehensive displacement experiments with unlabeled ligands. The Scatchard plots were linear in all three cases, suggesting the existence of a single binding site (curves not shown). The specific binding site of the new radioligand was determined in displacement binding assays. The inhibitory constants (K_i) are presented in **Table 6**.

μ-Ligands displaced [^{3}H][*(1S,2R)*ACPC]^{2}endomorphin-2 with the highest affinities, with a rank order of potency: DAMGO > naloxone > [(*1S,2R*)ACPC]^{2}endomorphin-2 >

endomorphin-2. Substitution of Pro^2 for *cis*-(*1S,2R*)ACPC^2 in endomorphin-2 resulted in a slight increase in the affinity in comparison with the parent ligand. The potencies of the κ-opioid selective NorBNI and U-50,488H were diverse. K_i (19.9 ± 6.1 nM) for the former compound was comparable to that reported for its binding to μ-opioid receptors (Porthogese et al., 1987), suggesting its low selectivity for the κ-opioid receptor. U-50,488H gave a 3 orders of magnitude higher inhibitory constant.

Table 6. Inhibitory constants (K_i) of μ-, δ-, and κ-specific opioid ligands against [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 on a rat brain membrane preparation.

Opioid receptor-subtype specific ligands	**K_i (nM)**
Naloxone (μ >> δ > κ)	0.98 ± 0.09
DAMGO (μ)	0.54 ± 0.08
Endomorphin-2 (μ)	2.8 ± 0.6
[(*1S,2R*)ACPC]^{2}endomorphin-2 (μ)	1.8 ± 0.4
Ile^{5,6}-deltorphin-2 (δ)	848 ± 19
DPDPE (δ)	337 ± 71
Naltrindole (δ)	29 ± 12
U-50,488H (κ)	398 ± 79
NorBNI (κ)	20 ± 6

K_i values were calculated according to the Cheng-Prusoff equation: $K_i = EC_{50}/(1+[ligand]/K_d)$ where K_d =1.8 nM was obtained from the isotope saturation curves. Results were obtained from the displacement curves. Data are expressed as means ± S.E.M., n ≥ 3.

The δ-specific peptide agonists Ile^{5,6}-deltorphin-2 and DPDPE were also found to be poor competitors of the tritiated ligand. Interestingly, the potency of the heterocyclic δ-specific antagonist naltrindole was only 16-fold lower than that of [*(1S,2R)*ACPC]^{2}endomorphin-2, reflecting the poor δ-selectivity of naltrindole.

5. Discussion

Despite the recent advances in the structural investigations and the understanding of the 3D features of the endomorphins, the bioactive conformations of these relevant tetrapeptides have remained unknown. To reveal the exact structural orientations and interactions between the μ-opioid receptors and the endomorphins, numerous analogs needed to be synthetized. The extensively examined *cis-trans* isomerization around the Tyr^1-Pro^2 backbone allows the endomorphins to adopt many accessible conformations, which make their structural properties more difficult to determine. In the present study, unnatural amino acids were introduced into the appropriate positions to generate conformational constraints, and proteolytic stability, and to gain more information about the possible bioactive structures of the endomorphins. In the course of the targeted structural modifications of these peptides, it has previously been shown that Dmt^1 substitution of the Tyr^1 residue may result in potent agonist or antagonist derivatives with high affinities and various selectivites (Balboni et al., 2002; Bryant et al., 2003; Okada et al., 2003; Tóth et al., 2004). This efficacy and variability may be due to the bonds and interactions between Dmt^1 and the remaining structural elements since the Tyr^1-Pro^2 and aromatic-aromatic interactions (Tyr^1/Dmt^1-Trp^3, -Phe^3, -Phe^4) have been found to contribute to the stabilization of the local conformations of endomorphins (In et al., 2001; Leitgeb 2007). Moreover, the incorporated methyl groups may appreciably diminish the acidity of the phenolic hydroxy group, thereby optimizing the hydrophobic and steric interactions of the analogs with the opioid receptors in such a way that the substituted analogs retain their affinities for the μ-sites, though they usually suffer a marked loss of selectivity as previously mentioned. Confirming the former hypothesis, ^{1}H-NMR studies and comparisons of the Tyr^1- and Dmt^1-containing endomorphins in DMSO-d_6 at 300 K have indicated differences in the *cis/trans* ratio of peptides around the Tyr^1-Pro^2 and Dmt^1-Pro^2 peptide backbones (Tóth et al., 2004). It has been found that under these experimental conditions $[Dmt]^1$endomorphin-1 and $[Dmt]^1$endomorphin-2 favor the *cis* conformation (*cis/trans* ratio 2:1) (Tóth et al., 2004), this conformational preference differing from that calculated for the parent peptides (higher *trans* ratio), on the basis of molecular modeling studies (In et al., 2001; Leitgeb 2007). When the results of ^{1}H-NMR measurements are taken into account, a reduction of binding potency would be expected,

but both analogs exhibit appreciable and comparable binding to endomorphins both in rat brain membrane and in mouse brain preparations (Tóth et al., 2002; Tóth et al., 2004). The interpretation of molecular modeling and ^{1}H-NMR data therefore demands caution.

To control the above mentioned *cis/trans* isomerization around the Tyr^1-Pro^2 peptide backbones, alicyclic β-amino acids have been introduced into the position of Pro^2. The incorporation of racemic alicyclic β-amino acids (*cis*-ACPC/ACHC or *trans*-ACPC/ACHC) yielded a series of peptide analogs with altered structural properties, proteolytic stabilities and pharmacological activities (Keresztes et al., 2008).

From a structure-activity aspect, restrained and unrestrained molecular dynamic (MD) simulations revealed that these peptide surrogates may exist in many more conformational states than those were detected by ^{1}H-NMR measurements in DMSO-d_6 (Keresztes et al., 2008). Various bend, turn and extended structures have been proposed as possible conformations. However, ^{1}H-NMR and MD simulation results revealed that the pharmacologically active ligands (compounds **2** and **13**) readily adopt bent or turn structures (β- and γ-turns) rather than extended ones, which may be important for receptor binding and recognition (Leitgeb et al., 2003). This is in accordance with previous findings on potent morphiceptin analogs with constrained topologies (Mierke et al., 1990; Eguchi et al., 2002), though several unique structural elements have also been observed as a consequence of the elongated peptide chains, due to the application of alicyclic β-amino acids. This elongation is believed to have less importance for folding and orientation of the pharmacophoric elements, which further confirms the role of Pro^2 as a stereochemical spacer, based on MD simulation studies (Keresztes et al., 2008). Secondary structural elements have been found to be stabilized through intramolecular H-bonds. The exceptions were the C_7-turn and C_{10}-turn, where such H-bonds cannot be formed. These structures are most probably stabilized through aromatic-aromatic or aromatic-CH interactions, which have been shown to stabilized local conformations (Yao et al, 1994; Leitgeb 2007). Furthermore, ^{1}H-NMR studies have demonstrated the predominance of one isomer found in the *trans* peptide backbone. Both findings correlated well with biological data (Gentilucci et al., 2004; Leitgeb 2007). Although ^{1}H-NMR and MD simulation of some of the *trans*-ACPC/ACHC^2-containing analogs has not been performed, pharmacological results on compounds **5, 16** and **20** strongly suggest

that these analogs may tend to adopt similar binding structures to those of the *cis*-(*1S,2R*)ACPC/ACHC^2-containing compounds. These results further confirm that the chirality and spatial orientation are more important features for determination of the biological activity than the ring size of the alicyclic β-amino acids, which did not exert a notable influence on the receptor binding.

In competitive binding experiments, a trend of adverse potencies was observed for the *cis*-ACPC/ACHC^2-containing analogs of endomorphins as compared with that suggested for the *cis*-ACPC-containing morphiceptin analog (Mierke et al., 1990). Compounds with *cis*-(*1S,2R*)ACPC/ACHC^2 residues were assessed to be as potent as the native ligands and more potent than the *cis*-(*1R,2S*)ACPC/ACHC^2-carrying ones. Potent diastereomers were additionally found among the *trans*-(*1R,2R*)ACPC/ACHC^2-containing derivatives. This was surprising because the *trans*-ACPC-containing analogs of morphiceptin were shown to be inactive in earlier binding experiments (Mierke et al., 1990; Yamazaki et al., 1991, 1993). Furthermore, as stated above, this result suggests that these derivatives may adopt similar bioactive conformations to those of the *cis*-(*1S,2R*)ACPC/ACHC^2-containing ones. The findings of competition experiments indicated that alicyclic β-amino acid substitution of Pro^2 in the endomorphins led to a considerable reduction of the μ- *vs.* δ-receptor selectivity, as for the Dmt^1-containing analogs (Tóth et al., 2004). The comprehensive characterization of [^{3}H][(*1S,2R*)ACPC]^{2}endomorphin-2 has also confirmed the μ-receptor selectivity when measured against δ- and κ-selective ligands. Its favorable properties, such as the proteolytic stability and high specific activity, make it a very valuable research tool for the investigation of ligand-mediated processes.

The opioid receptors belong in the GPCR superfamily and are coupled to the pertussis toxin-sensitive G_i/G_o G-protein family, thus allowing investigation of receptor-ligand interactions through the widely-known [^{35}S]GTPγS functional binding assay. As this assay measures the level of G-protein activation following agonist or antagonist occupation of the binding site, its advantage is that it follows the direct functional consequences of receptor occupancy, independently of the downstream-effector systems. The results of [^{35}S]GTPγS binding experiments clearly demonstrated that alicyclic β-amino acid substitutions do not have pronounced effects on the agonist/antagonist

properties. The examined analogs mostly retained their partial agonist profiles, similar to those of the endomorphins. The joint analysis of the equilibrium competition, [^{35}S]GTPγS binding and ^{1}H-NMR studies and MD simulations suggest that the receptor-ligand interactions are governed by diverse structural aspects after the introduction of alicyclic β-amino acids. In order to explain the variability of the binding results, it is assumed that the opioid receptors might have different affinity states, which may be generated by either the *cis* or *trans* isomers of the opioid ligands bound to the receptors. Since the receptors reportedly exist in different local conformational states, ligands with either a *cis* or a *trans* conformation are capable of coupling to the receptors, but with distinct affinities and selectivities, depending upon their structural features. Another explanation may be the homo-oligomerization of opioid receptors and hetero-oligomerization of μ- and δ-opioid receptor types that have been documented in numerous reports, and are of particular interest, because these processes may entail profound changes in ligand binding and/or signaling (George et al., 2000; Gomes et al., 2000; Ho et al., 2001; Gomes et al., 2004; Snook et al., 2006). In view of these literature data, it seems likely that these phenoma may contribute alone or together to the complexity of the receptor-ligand interactions.

6. Summary

- A set of Dmt[1]- and/or ACPC/ACHC[2]-containing endomorphin analogs were synthetized to obtain proteolytically stable, conformationally constrained peptide analogs for structure-activity studies.
- Systematic receptor-binding studies of these derivatives revealed that substitutions by these amino acid surrogates result in equipotent or less potent compounds, with the loss of μ-receptor selectivity, as compared with the parent endomorphins, depending on the chiralities of the incorporated alicyclic β-amino acids.
- On the basis of *in vitro* receptor-binding studies, two potent isomers were selected for tritium labeling and comprehensive receptor binding characterization to obtain proteolytically stable, labeled peptide analogs. Both radioligands displayed high stability against self-decomposition during long-lasting storage. One of these labeled compounds, [^{3}H][*(1S,2R)*ACPC]^{2}endomorphin-2 has become commercially available research tool marketed by the Institute of Isotope Co. Ltd. (Budapest)
- Ligand-stimulated [^{35}S]GTPγS functional studies showed that alicyclic β-amino acid substitutions do not influence the efficacy profile, i.e. the analogs mostly retain their partial agonist properties.
- A combined analysis of the receptor binding, ^{1}H-NMR and MD simulation data revealed that the four examined *cis*-ACPC/ACHC[2]-containing analogs have a tendency to form a bent, folded structure rather than an extended one.

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2.2. Significance of Chirality of Alicyclic β-Amino Acids and of Isomerism in Endomorphins

The *cis-trans* isomerization of the Tyr-Pro peptide bond seems to be a relevant structural feature that has significant influence on the bioactivity of the endomorphins. It defines a free rotation around the omega Tyr-Pro peptide bond and results in numerous secondary structures. The energy difference between the *cis*- and *trans*-conformers is considerably low (2-4 kcal/mol), which allows reversible interconversion between the two isoforms. The determination of the relative proportions of the *cis-trans* isomers of endomorphins faces several problems. The ratio of the two conformers strongly depends upon the media applied and the variety of experimental techniques. The majority of findings reports on a 25:75 *cis/trans* ratio with a few exception, measured in DMSO, water and micelles using ^{1}H-NMR and theoretical calculations. This ratio seems to be uniformly valid for both endomorphins and entails diverse pharmacological activities. A reverse ratio of conformers was observed by substituting the pharmacophore Tyr^{1} with dimethyltyrosine (Dmt). The introduction of two methyl groups into the tyrosine restrains the free rotation of the omega peptide bond, and forces the structure into new conformations. Although, the omega peptide bond, the α-carbon atoms and the side-chain conformations of the residual amino acids contribute altogether to the actual conformation of endomorphins, the substitutions of the natural amino acids located in the position 3 and 4 did not result in significant changes of conformations, measured in DMSO. These facts suggested that the Tyr^{1} and Pro^{2} residues have a crucial role in determining the bioactive conformation of endomorphins. Due to the isomerism and the secondary interactions, both peptides in *cis*- and *trans* conformations, may occur in folded, bent as well as in extended conformations, which further complicate the 3D-structures of endomorphins.

The alicyclic β-amino acids are of a huge importance as a consequence of their unique structural properties. In these peptides, the amino and carboxyl functions are located on neighbouring carbon atoms, and thus, exist as *R* or *S* isomers with a total

number of four possible enantiomers (*R,R; R,S; S,S or S,R)*. (The *R/S* abbreviation labels the chiral centers, and is the most important nomenclature system for denoting enantiomers. Its major advantage is that it does not require reference molecule). The incorporation of these molecules into bioactive peptides provides diastereomers as a results of the two chiral centers. (Diastereomers are stereoisomers that are not related as object and mirror image). The stereo- and regio-isomers, together with the possible ring size expansions and further substitutions on the ring, significantly increase the structural diversity of alicyclic β-amino acids. The insertion of an alicyclic β-amino acid into the place of a natural amino acid is currently believed to improve both the biological activity and the proteolytic stability.

Alulírott **…….** hozzájárulok, hogy **Keresztes Attila** felhasználja

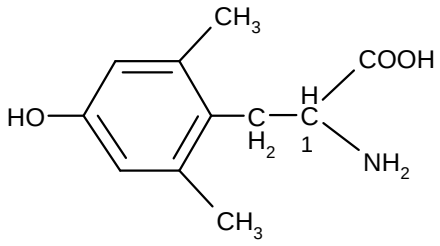
(publikációs lista)

közleményeinkben/közleményünkben foglalt eredményeinket a Szegedi Tudományegyetem Elméleti Orvostudományi Doktori Iskola keretében a PhD fokozat eléréséért benyújtott dolgozatában, és egyúttal kijelentem, hogy ezeket az eredmény(eke)t nem használtam fel és nem is kívánom felhasználni sem a Szegedi Tudományegyetem, sem másutt az elméleti orvostudományok tudományszakon PhD fokozat megszerzéséhez.

Szeged, 2007.08.31.

(aláírás)

2,6-dimethyl-tyrosine (Dmt)



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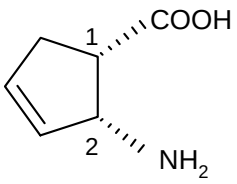

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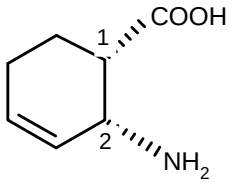

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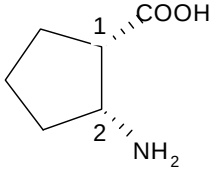

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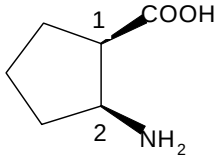

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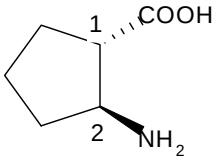

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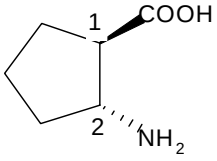

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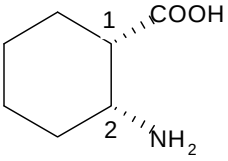
2-aminocyclohexanecarboxylic acid (ACHC)



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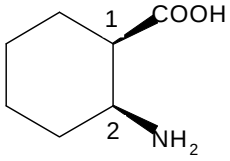

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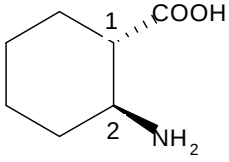

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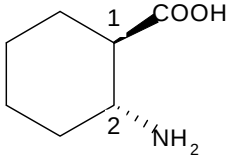

trans- (1*R*,2*R*)-

Fig. 1 Representative chemical structures of the incorporated unnatural amino acids. The application of the alicyclic β-amino acid ACPC and ACHC resulted in diastereomer pairs as a consequence of the two chiral centers, while Dmt provided only one corresponding analog.

Peptides	**Half-life**
endomorphin-1	4.9 ± 0.2 min.
[(*1S,2R*)ACPC]^{2}endomorphin-1	> 12 h
[(*1S,2R*)ACHC]^{2}endomorphin-1	> 12 h
endomorphin-2	3.8 ± 0.2 min.
[(*1S,2R*)ACPC]^{2}endomorphin-2	> 12 h
[(*1S,2R*)ACHC]^{2}endomorphin-2	> 12 h

Table 1. Classification of endogenous opioid peptides

Receptor	Precursor Protein	Endogenous peptide	Amino Acid Sequence	Natural Source	References
μ (MOR)	Proopiomelanocortin	β-endorphin	YGGFMTSEKQTPLVTLFKN AIIKNAYKKGE	Mammalian brain	*Li and Chung (1976)*
	β-Casein (bovine)	β-Casomorphin-5 β-Casomorphin-7 Morphiceptin	YPFPG YPFPGPI YPFP-NH_2	Bovin milk	*Blanchard et al. (1987)*
	β-Casein (human)	β-Casomorphin-5 β-Casomorphin-7	YPFVE YPFVEPI	Human milk	*Blanchard et al. (1987)*
	Hemoglobin	Hemorphin-4 Hemorphin-7	YPWT YPWTQRF	Human blood	*Nyberg et al. (1997)* *Zhao et al. (1997)*
	Unknown	Dermorphin Endomorphin-1 Endomorphin-2 Tyr-MIF-1 Tyr-W-MIF-1	YaFGYPS-NH_2 YPWF-NH_2 YPFF-NH_2 YPLG-NH_2 YPWG-NH_2	Frog skin Bovine and human brain	*Bozu et al. (1997)* *Zadina et al. (1997)* *Zadina et al. (1994)*
δ (DOR)	Proenkephalin	[Met^5]enkephalin [Leu^5]enkephalin	YGGFM YGGFL	Mammalian brain	*Hughes et al. (1975)*
	Unknown	Dermenkephalin Deltorphin-1 Deltorphin-2	YmFHLMD YaFDVVG-NH_2 YaFEVVG-NH_2	Frog skin	*Amiche et al. (1989)* *Kreil et al. (1989)*
κ (KOR)	Prodynorphin	Dynorphin A Dynorphin A(1-8) Dynorphin B	YGGFLRRIRPKLKWDNNQ YGGFLRRI YGGFLRRQFKVVT	Mammalian brain	*Chavkin et al. (1982)*
ORL_1 (NOP)	Pronociceptin	Nociceptin/orphanin FQ	FGGFTGARKSARKLANQ	Mammalian brain	*Meunier et al. (1995)* *Reinscheid et al. (1995)*