Design, Synthesis and Pharmacological Evaluation of Novel Endomorphin Analogues with Multiple Structural Modifications

Ph.D. Thesis

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Chemical Ph.D. School

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List of Abbreviations

ΔAcpc	2-aminocyclopent-3-enecarboxylic acid
Acpc	2-aminocyclopentanecarboxylic acid
ΔAchc	2-aminocyclohex-6-enecarboxylic acid
Achc	2-aminocyclohexanecarboxylic acid
ACN	acetonitrile
AcOH	acetic acid
Aia	4-amino-1,2,4,5-tetrahydro-indolo[2,3-c]-azepin-3-one
Aib	2-aminoisobutyric acid
BBB	blood-brain barrier
Boc	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
Cha	cyclohexylalanine
Ci	curie
CNS	central nervous system
°C	degrees centigrade
DAMGO	H-Tyr-D-Ala-Gly-NMePhe-Gly-ol
DCC	N,N-dicyclohexylcarbodiimide
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
Dmt	2',6'-dimethyltyrosine
DOR	δ-opioid receptor
EGTA	ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*,*N*-tetra acetic acid
ESI-MS	electrospray ionization mass spectrometry
EC_{50}	half maximal effective concentration
EM-1	endomrrphin-1: H-Tyr-Pro-Trp-Phe-NH_2

EM-2	endomorphin-2: H-Tyr-Pro-Phe-Phe-NH_2
EtOH	ethanol
EBA	evans blue labeled albumin
GITC	2,3,4,6-tetra-O-acetyl-β-glucopyranosyl isothiocyanate
GDP	guanosine-5'-diphosphate
GPCR	G-protein-coupled receptor
GTP	guanosine-triphosphate
GTPγS	guanosine-5′-*O*-(3-thio)triphosphate
Hfe	homophenylalanine
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry
Hyp	(2*S*,4*R*)-hydroxyproline
i.c.v	intracerebroventricular
i-PrOH	iso-Propanol
$Ile^{5,6}$-deltorphin-2	H-Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2
Inp	piperidine-4-carboxylic acid
i.t	intrathecal
K_d	equilibrium dissociation constant
K_i	inhibitory constant
KOR	κ-opioid receptor
MBHA	4-methylbenzhydrylamine
MeOH	methanol
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MOR	μ-opioid receptor
MS	mass spectrometry
Δ^zPhe	(*Z*)-*α*, β-didehydrophenylalanine
βMePhe	β-methylphenylalanine
βPro	β-homoproline
Nip	piperidine-3-carboxylic acid
NMR	nuclear magnetic resonance spectroscopy

NOE	nuclear Overhauser effect
NVA	norvaline
pFPhe	para-fluorophenylalanine
PAL	peptidylamidoglycolate lyase
PAM	peptidylglycine α-amidating monooxygenase
Phg	phenylglycine
PHM	peptidylglycine-α-hydroxylating monooxygenase
Pip	piperidine-2-carboxylic acid
Pra	propargylglycine
ΨPro	pseudoproline
R_f	retention factor
ROESY	rotating-frame Overhauser effect spectroscopy
RP-HPLC	reversed-phase high-performance liquid chromatography
TBq	tera Becquerel
TEA	triethylamine
TFA	trifluoroacetic acid
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TLC	thin-layer chromatography
TM	transmembrane
TOCSY	total correlation spectroscopy
Tris	tris-(hydroxymethyl)-aminomethane
Tyr-W-MIF	H-Tyr-Pro-Trp-Gly-NH_2
SAR	structure activity relationship
SF	sodium fluorescein
UFP-12	Phe-Gly-Gly-pFPhe-Thr-Gly-Aib-Arg-Lys-Ser-Ala-Arg-Lys-Arg-Lys-Asn-Gln-OH

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1. Introduction

1.1. The opioid system

Pain is a highly subjective, unique sensation. Opium has been extracted from the poppy plant and used to treat pain for thousands of years but with little understanding of its mechanism of action. The major active ingredient of opium is the alkaloid morphine, which was first isolated by Wilhelm Sertürner in 1805. The discovery of morphine was considered a milestone in medical history. Despite its severe side effects like respiratory depression, severe drowsiness, weakness, dizziness, slurred speech, nausea or vomiting, physical dependence and constipation etc., it's still being used as a medicine for pain relief.

Opioid receptors were first proposed in 1954 by Beckett and Casy by demonstrating the existence of specific sites for opioid ligands, which was a breakthrough in biochemical pharmacology [1]. Opioid receptors, mediating the actions of opiate alkaloids and opioid peptides, belong to the superfamily of G-protein-coupled receptors (GPCRs). These receptors mediate a wide variety of physiological functions including neurotransmission, hormone- and enzyme release from endocrine and exocrine glands, immune responses, cardiac- and smooth-muscle contraction and blood pressure regulation [2]. To date, three types of opioid-binding sites have been identified based on pharmacological and behavioral studies, namely μ- (MOR), δ- (DOR), and κ- (KOR) receptors. Although, only single genes for MOR, DOR and KOR have been cloned, the opioid-binding sites do not form homogenous populations [3].

Opioid receptors belong to the membrane-bound class A (Rhodopsin) family of G_i/G_o protein coupled receptors composed of a single polypeptide chain with seven transmembrane (TM) domains with an extracellular N-terminal and an intracellular C-terminal domain. These receptors are about 60-70% identical to each other with the greatest homology in the TM helices, the intracellular loops, and a portion of the C-

terminal tail adjacent to the TM7 domain and the greatest diversity in their N- and C-termini as well as their extracellular loops [4]. The extracellular N-terminus of the receptor proteins have varying number of glycosylation sites, [5] the intracellular C-terminus can be modified post-translationally by fatty acids and it contains multiple phosphorylation sites. Both termini with the extracellular loops have been shown to be highly diverse in amino acid sequences [6].

Opioid receptors are widely distributed throughout the brain and periphery [7]. Higher μ-receptor density has been found for the thalamus, caudate putamen, neocortex, periaqueductal gray and the dorsal horn of the spinal cord [8]. The olfactory bulb, neocortex, caudate putamen, nucleus accumbens and amygdala are rich in δ-receptors. κ-receptors are observed in areas of the cerebral cortex, nucleus accumbens, claustrum and hypothalamus [7].

The exo- and endo opioid ligands regulate the nociceptive pathway that includes pain perception, modulation and the response to painful stimuli. In addition to this, opioid ligands mediate respiratory, cardiovascular, gastrointestinal, renal, and hepatic functions, mood and feeding behavior. Clinical applications of opioid ligands are impeded due to the side-effects such as the development of tolerance and dependence [7, 9, 10 and 11]. The ligands and their precursor proteins for μ-, δ- and κ-receptors have been investigated thoroughly. β-Endorphin has shown equal activity at μ- and δ-receptors with a much lower affinity for κ-receptors [12]. Met- and Leu- enkephalins have high affinities for δ-receptors and lower affinities for μ-receptors [13]. The opioid fragments of pro-dynorphin, particularly dynorphin A and dynorphin B, have high affinity for κ-receptors but also have significant affinity for μ-and δ-receptors [14]. Nociceptin/Orphanin FQ is the endogenous ligand for the ORL_1-receptor; it has little affinity for the μ-, δ- and κ-receptors [15, 16 and 17]. 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	*12*
	β-Casein (bovine)	β-Casomorphin-5	YPFPG	Bovine milk	*18*
		β-Casomorphin-7	YPFPGPI		
		Morphiceptin	YPFP-NH_2		
	β-Casein (human)	β-Casomorphin-5	YPFVE	Human milk	*18*
		β-Casomorphin-7	YPFVEPI		
	Hemoglobin	Hemorphin-4	YPWT	Human blood	*19*
		Hemorphin-7	YPWTQRF		*20*
	Prodermorphin	Dermorphin	YaFGYPS-NH_2	Frog skin	*21*
	Unknown	Endomorphin-1	YPWF-NH_2	Bovine and human brain	*22*
		Endomorphin-2	YPFF-NH_2		
		Tyr-MIF-1	YPLG-NH_2		*23*
		Tyr-W-MIF-1	YPWG-NH_2		
δ (DOR)	Proenkephalin	[Met^5]enkephalin	YGGFM	Mammalian brain	*13*
		[Leu^5]enkephalin	YGGFL		
	Prodermorphin	Dermenkephalin	YmFHLMD	Frog skin	*24*
		Deltorphin-1	YaFDVVG-NH_2		*25*
		Deltorphin-2	YaFEVVG-NH_2		
κ (KOR)	Prodynorphin	Dynorphin A	YGGFLRRIRPKLKWDNNQ	Mammalian brain	*14*
		Dynorphin A(1-8)	YGGFLRRI		
		Dynorphin B	YGGFLRRQFKVVT		
ORL_1 (NOP)	Pronociceptin	Nociceptin/orphanin FQ	FGGFTGARKSARKLANQ	Mammalian brain	*15-17*

1.2. Effector mechanisms

When an agonist ligand (morphine, enkephalin, N/OFQ etc) binds to the receptor, it induces a conformational change in the receptor, consequently receptor proteins become activated with the exchange of guanine nucleotides in the G_α-subunit. Upon activation, it relays the signal to other components in the downstream signaling cascade. Activation of the G_α subunit of regulatory G-proteins (G_i/G_q and G_o types mainly) is the key factor in opioid signaling within the membrane [11, 18]. Initially, it was thought that μ- and δ-receptors coupled through G_i/G_o proteins activate an inwardly rectifying potassium channel and inhibit voltage-operated calcium channels whereas κ-receptors only inhibit voltage-operated calcium channels. Later it was shown, that all of the opioid receptors most likely share common effector mechanisms [19, 20]. Activation of any type of opioid receptor inhibits adenylate cyclase, resulting in a fall in intracellular cAMP and diminished action potential firing. This causes a reduced flow of nociceptive information to the brain [11]. Conversely, opioid addicts undergoing withdrawal suffer elevated cAMP levels and enhanced protein kinase A activity, resulting in an increased neurotransmitter release [26].

1.3. Development and clinical applications of opioid ligands

Opioid receptors are unique in that their existence was proposed before the discovery of the natural agonists [1]. Opioid alkaloids morphine, codeine and thebaine were considered as "natural-product hits" on which chemical programmes were based to design analogues with an improved pharmacology. Since morphine administration is associated with undesirable side effects, great scientific attention is directed towards the design of modified opioid peptides which maintain an ability to bind to μ-opioid receptors with high affinity and selectivity and good penetration through the blood-brain-barrier (BBB). The discovery of the enkephalins of the δ-receptor, evoked the idea to synthesize a new class of opioid agonists with a lack of addictive properties of morphine. [27]. Such synthetic products are being tested in animal models, and the development of

a selective non-peptide agonist is under consideration by a number of commercial drug houses. Preclinical studies suggest that δ-agonists may have a superior profile as analgesics, but this will only be established when such an agent is successfully introduced into clinical investigation; other possible applications of selective ligands for this receptor may emerge from clinical experience. The development of opioid ligands for κ-opioid receptor analgesics was based on the preclinical pharmacology of the 6,7-benzomorphans such as ketazocine and its derivatives. Although these products elicited powerful antinociceptive effect, they are not a substitute for morphine. The clinical utilities of agonists or antagonists for the ORL_1 receptor can only be the subject of speculation. [28].

1.4. Blood-Brain Barrier (BBB) and its significance

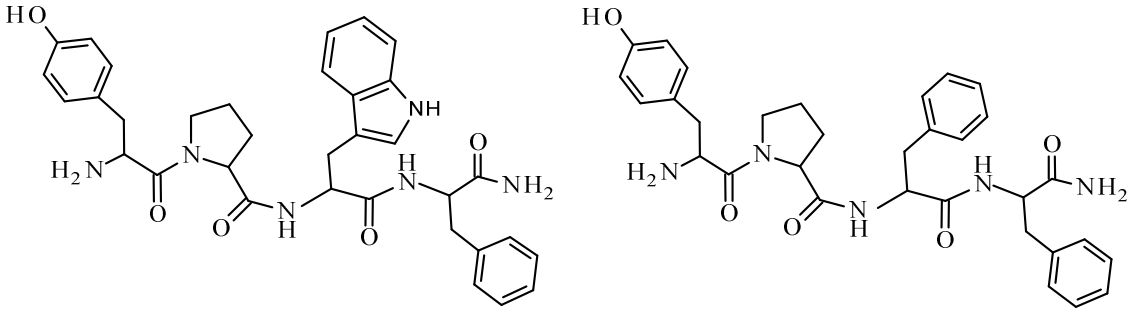
The BBB is an active interface between the circulation and the central nervous system (CNS) which confines the free movement of different substances between the two compartments and plays a crucial role in the maintenance of the homeostasis of the CNS. The BBB has a dual role, a barrier and a carrier function. The barrier function restricts the transport from the blood to the brain of potentially toxic or harmful substances; the carrier function is responsible for the transport of nutrients to the brain and removal of metabolites. The relative impermeability of the barrier not only protects the brain from potentially harmful substances, but also prevents different drugs to reach therapeutically relevant concentrations in the brain [29]. The BBB has a significant importance in the clinical practice as well. On one side there are a large number of neurological disorders including cerebral ischemia, brain trauma and tumors, neurodegenerative disorders, in which the permeability of the BBB is increased. On the other hand due to the relative impermeability of the barrier many drugs are unable to reach the CNS in therapeutically relevant concentration, making the BBB one of the major hindrances in the treatment of CNS disorders [30]. Most of the native opioid peptides in general have a limited *in vivo* efficacy, due to their poor metabolic stability and limited delivery to the CNS [31].

Therefore it is of great importance to design opioid peptide analogues with improved peptidase resistance and greater BBB permeation.

1.5. Discovery of endomorphins

In 1997, J .E. Zadina and coworkers synthesized a number of Tyr-W-MIF (H-Tyr-Pro-Trp-Gly-NH_2) with substitution of possible natural amino acids in position 4 by using combinatorial chemistry and subsequently screened for their μ-opioid receptor binding Two ligands were found with high affinity and selectivity toward the μ-opioid receptor. These tetrapeptides were isolated initially from bovine brain [22], and subsequently from human brain cortex [32] and were named endomorphin-1 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2). Schematic chemical structures of endomorphins are shown in **Figure 1**. Endomorphins were the first peptides isolated from brain that bind to the μ-opioid receptor with high affinity and selectivity and therefore were proposed as endogenous μ-opioid receptor ligands [22]. Their precursor(s) or the possible biosynthetic route(s) still remains unidentified, whereas the biosynthetic pathways for other vertebrate opioid peptides have already been clarified [33]. Concerning their anatomical distribution, EM-1 is widely and densely distributed throughout the brain and upper brainstem and is particularly abundant in the nucleus accumbens, the cortex, the amygdala, the thalamus, the hypothalamus, the striatum, and the dorsal root ganglia [34, 35]. Endomorphin-2 is more prevalent in the spinal cord and lower brainstem and in the dorsal horn of the spinal cord [34, 36 and 37]. Endomorphins display notable binding affinities (K_i = 0.3 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, and also interesting pharmacological and structural features. In *in vivo* experiments endomorphins turned out to be as effective as the majority of opioid peptides targeting the μ-opioid receptors, even in low doses exhibited outstanding potencies toward both intractable acute and chronic neuropathic pain. Intracerebroventricular (i.c.v) and intrathecal (i.t) administration of endomorphins evoked sustained antinociception in wild type adult rodents in tail-flick, paw-withdrawal and tail-pressure tests [22, 38, 39, 40, 41,

42, 43, 44 and 45]. Endomorphins and morphine are alike in binding to the μ-opioid receptor, but endomorphins are thought to inhibit pain with fewer side effects [46].



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

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

Figure 1. *Schematic chemical structures of the endomorphins*

1.6. Structural modifications of endomorphins

For the classic endogenous opioid peptides, like enkephalins, dynorphins, endorphins the N-terminal Tyr-Gly-Gly-Phe sequence confers the "message sequence", while the remaining C-terminal constitutes the "address sequence" [47]. It was initially thought that in endomorphins the "message sequence" consists of two pharmacophore amino acids, the Tyr^1 and Trp^3/Phe^3 residues which are essential for the μ-opioid receptor recognition and binding, while the "address sequence" is found to be responsible for μ-opioid receptor vs δ-opioid receptor selectivity [48]. Later it was found that not the whole "message sequence" but the phenolic group of Tyr^1 and the aromatic side chain at position 3 are essential for the binding of endomorphins to μ-opioid receptors. In the "address sequence" the C-terminal aromatic side chain and amine functions have been found to be responsible for μ- vs δ-opioid receptor selectivity of endomorphins [49].

Hence endomorphins bind to the μ-opioid receptor with high affinity they have drawn substantial attention from a pharmacological aspect. Upon exogenous application, endomorphins suffer from serious limitations including short duration of action, lack of activity after oral administration, relative inability to cross the BBB into the central nervous system (CNS) and poor metabolic stability [31, 50 and 51]. To overcome aforementioned difficulties/restrictions, a systematic modification of the peptide sequence is required.

The substitution of Tyr^1 with Dmt resulted in an increased affinity not only for the μ-, but also for the δ-receptors [52, 53, 54 and 55]. Furthermore, incorporation of N-allyl-Dmt^1 in EM-1 and -2 resulted in neutral μ-opioid antagonists, these analogues inhibited the naloxone/naltrexone-elicited withdrawal syndromes without adverse effects observed with inverse agonist alkaloid-derived compounds [56, 57]. But, the substitution of N, N-allyl-Dmt^1 yielded a decreased μ-opioid receptor affinity which suggests that the presence and the conformation of Tyr^1 is crucial for interaction with opioid receptors.

Proline in the second position of endomorphins is considered a spacer residue, connecting two pharmacophoric aromatic residues, Tyr^1 and Trp^3/Phe^3 [47]. Incorporation of Acpc/Achc residues into the place of Pro^2 in endomorphins yielded analogues with increased proteolytic stability and binding ability depending on the configuration of the incorporated alicyclic β-amino acid [58, 59]. Replacement of Pro^2 with Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) in endomorphins resulted in dual properties, acting as a μ-receptor agonist/δ-antagonist. This is remarkable because mixed μ-agonist/δ-antagonist ligands are assumed to have a higher tendency to suppress the development of analgesic tolerance [60]. Furthermore, insertion of pseudoproline (ΨPro) in place of Pro^2 in EM-2 analogues, retained μ-opioid receptor affinity and selectivity [61]. Additionally, six-membered heterocyclic rings, such as piperidine-2-, 3- and 4-carboxylic acids [(*S*)-Pip, (*R*)-Nip and Inp, respectively] were also incorporated into the place of Pro^2 in EM-2. [(*R*)-Nip^2]EM-2 displayed higher proteolytic stability and increased affinity for the μ-opioid receptor compared to the parent peptide in rat brain

homogenate [62]. These results confirm that Pro^2 targeting modifications yield proteolytically stable analogues.

Substitution of Phe^3 with cyclohexylalanine (Cha) residue, which lacks aromaticity and a quadrupole moment, led to decreased affinities relative to EM-2, suggesting that the presence of π electrons or the planarity of the aromatic ring is necessary for the ligand-receptor interaction [63]. Incorporation 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 analogues with decreased binding potencies [63-65]. Observations of these results suggest that in addition to the presence of an aromatic ring at position 3, the steric situation of the side chain determines the outcome of the receptor-ligand interaction.

The insertion of $pFPhe^4$ instead of Phe^4 in enkephalin yielded increased potency in functional assays [66]. Similar results were observed in endomorphins as well [67]. Tömböly and co-workers observed remarkable differences in biochemical properties of the βMePhe substituted endomorphins with different stereochemistry in the position of Phe^4 in EM-1 and Phe^3/Phe^4 in EM-2. Substitution of $\beta MePhe^4$ resulted in increased affinity and potency in EM-1 and -2, but the substitution of Phe^3 by βMePhe in EM-2 resulted in a loss of μ-opioid receptor affinity which confirmed that the proper orientation of Phe^4 aromatic side chain is a crucial part of the address sequence for μ-opioid receptor activity [68]. Chemical modification of the C-terminal part of the endomorphins, including replacement of the carboxamide group by hydroxymethyl (CH_2-OH), methylester (COOMe) or hydrazide (CO-$NHNH_2$) led to similarly potent analogues compared to the parent ligands [69]. The above detailed structural modifications are summarized in **Table 2**.

Table 2. Some typical structural modifications performed on endomorphins

Amino acids	Inserted position in the sequence	Effects of substitution on endomorphins	References
2,6-dimethyltyrosine (Dmt)	Tyr^1	Increased binding potency and decreased receptor selectivity	*52-55*
N-allylation of the N-terminal 2,6-dimethyltyrosine (N-allyl-Dmt)	Tyr^1	Decreased μ-opioid receptor affinity	*56-57*
2-Aminocyclopentanecarboxylic acid (Acpc), 2-Aminocyclohexanecarboxylic acid (Achc)	Pro^2	Increased, decreased or unaltered receptor affinity with decreased μ-opioid receptor selectivity depending on chirality of alicyclic β-amino acid	*58, 59*
1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (Tic)	Pro^2	Dual properties, acting as a μ-receptor agonist/δ-antagonist	*60*
Piperidine-2- carboxylic acid (Pip) Piperidine-3- carboxylic acid (Nip) Piperidine-4- carboxylic acid (Inp)	Pro^2	Nip substitution resulted in the most potent analogues	*62*
Cyclohexylalanine (Cha) 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	*63, 65, 70*
para-fluoro phenylalanine (pFPhe) β-methylphenylalanine (βMePhe)	Phe^4	Increased potencies and efficacies	*66, 68*
Phenylalanine with carboxamide, hydroxymethyl, methylester, hydrazide	Phe^4	Similar potency to the parent ligands	*69*

1.7. Stability of endomorphins

Opioid peptides easily undergo rapid enzymatic degradation soon after their production. Most of the peptide-degrading enzymes are membrane-bound exo- and endopeptidases, which have active sites facing to the extracellular space. Hence the majority of degradation processes of opioid peptides take place in the extracellular space. However, an intracellular cleavage of opioid peptides after internalization by cytosolic peptidases cannot be excluded [71]. Peptide-degrading enzymes are able to split off the backbones of the endomorphins. Among the known mammalian opioid peptides, endomorphins have the longest half-lives (4-6 min) due to the presence of the proline residue in the second position, which not only influences the conformation of the peptide but also confers the stability against most proteases [31, 72 and 73]. The metabolic pathway of endomorphins has been mapped and summarized in a recent review [71]. *In vitro* metabolic studies demonstrated that the main cleavage occurs at Pro^2-Trp^3 and Pro^2-Phe^3 peptide bonds; the dipeptides thus formed are then hydrolyzed into individual amino acids. Two enzymes in particular are responsible for initiating degradation of endomorphins. Dipeptidyl peptidase IV, a membrane bound serine protease and carboxypeptidase Y (producing the carboxy end), activate the process and aminopeptidases terminate the reaction [51, 74, 75 and 76]. The total degradation takes approximately 1 h for EM-1 and 2-3 hours for EM-2 [73, 76]. However, it has also been reported that EM-1 might be more resistant to proteolytic degradation, as it produced a longer duration of spinal antinociception compared to EM-2. The effects of protease inhibitors on degradation of endomorphins clearly indicated the role of inhibitors such as actinonin, diprotin A and Ala-pyrrolidonyl-2-nitrile in delaying or modifying the peptide potency by blocking endomorphin metabolism either *in vivo* or *in vitro* [74]. The activity of endomorphin degradation products has been examined. The obtained results revealed that the metabolic products are pharmacologically inactive; subsequently the analgesic activity is expected to be diminished within minutes of endomorphin degradation [77].

1.8. Importance of chirality of alicyclic β-amino acids and isomerism in endomorphins

Due to their unique structural properties, alicyclic β-amino acids have a significance importance in constructing biologically active substances [78]. In these amino acids, the amino and carboxyl functions are located on neighboring atoms. The two chiral centers offer four possible stereoisomers, two configurational *cis*-(*R,R* and *R,S)* and two configurational *trans*-*(S,S* and *S,R)*. Incorporation of these racemic mixtures into bioactive peptides allows for diastereomers. (Diastereomers are stereoisomers that are not mirror images of one another and are non superimposable on one another). The stereo- and regio-isomers, together with the possible ring size expansions and further substitutions, significantly extend the structural diversity of alicyclic β-amino acids [78]. The insertion of an alicyclic β-amino acid in place of Pro^2 yields peptides with increased proteolytic stability while retaining or enhancing the biological activity depending on the chirality of amino acid [58, 79].

Hence endomorphins contain Pro in position 2, they exist as an equilibrium mixture of *cis*- and *trans*-conformations [48] and the isomerization around the Tyr^1-Pro^2 peptide bond has been reported by means of various different techniques [48, 49,80, 81, 82 and 83]. Due to the sufficient low energy (2-4 kcal/mol) difference between the conformers allows the formation of either *cis*- or *trans*- conformers with respective population rates [48, 49]. The ^{1}H-NMR spectroscopic data and SAR studies measured in DMSO, water and SDS micelles indicated that the *cis*/*trans* ratio was 1:3 and 1:2 for EM-1 and -2, respectively [48, 81 and 83]. This balance of configurations has been shown to be alerted by incorporation of Dmt and pseudoproline (ψPro) in positions 1 and 2, respectively [53, 61].

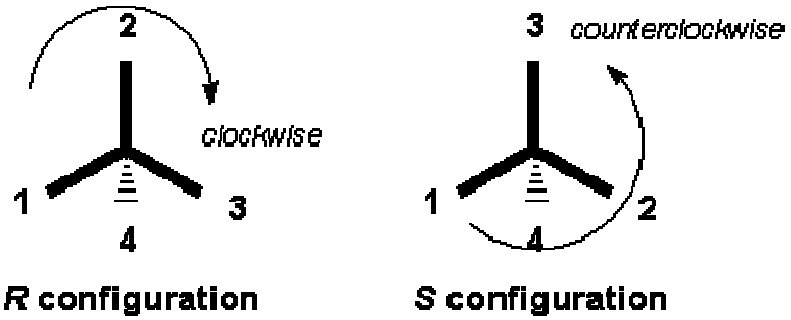
Numerous experiments have been carried out in search of bioactive conformations of endomorphins, but none provided emphatically convincing models for the biologically relevant conformations of peptides. Depending on the experimental conditions the ratio of the two conformers varied, as they easily changed from one

conformer to another, yielding a series of conflicting results. Furthermore, these short tetrapeptides can adopt compact or extended structures in either *cis*- or *trans*-conformation and this further elicits the number of possible 3D-structures [49, 81]. Consequently, the bioactive conformations of endomorphins remained an unanswered question. However, a slightly bent backbone structure was proposed for receptor-bound ligands, more recently four structural parameters were confirmed for high μ-opioid activity. Those include (1) *trans*-conformation of N-terminal aromatic side chain, (2) the preference for *gauche*– conformation of aromatic side chain in the third position, (3) flexibility of the C-terminal aromatic moiety and (4) bent backbone structure of the peptide [84, 85].

Brief description of *R* & *S* notation

R- and *S*- notation use the Cahn-Ingold-Prelog (CIP) priority rules [86] for the assignment of the *absolute configuration* around a stereocenter.

Using CIP rules assign the priorities of the each bonded molecule surrounding the stereocenter. A clockwise direction is an *R* (latin: *rectus*) configuration. A counterclockwise direction is an *S* (latin: *sinister*) configuration.



Direction of the travel 1-2-3 dictates configuration.

If least priority group present at dark position then stereochemistry reverse.

1.9. Pharmacology of endomorphins

The potent antinociceptive behavior of endomorphins after i.c.v or i.t injection is blocked by μ-opioid antagonists and is diminished in μ-opioid receptor knockout mice. The results suggest that endormorphins preferentially bind to μ-opioid binding sites [9, 22, 87 and 88]. Both peptides present similar affinities, but greater selectivities than the μ-opioid full agonist DAMGO or morphine. Neither of the compounds had significant affinity for δ- or κ-opioid receptors [9]. In competitive radioligand receptor binding assays, endomorphins displace the general antagonist naloxone, DAMGO and other μ-opioid selective ligands in a concentration dependent manner [9]. It has been suggested that endomorphins exert their pharmacological activities by stimulating two diverse subtypes of hypothetical μ-opioid receptors, μ_1 and μ_2. Subcutaneous application of the μ_1-receptor-selective antagonist naloxonazine prevents the antinociceptive and antitransit action of EM-1. Moreover these effects are partially blocked by i.c.v. naloxone or by i.p. naloxone methiodide. The δ-opioid receptor antagonist naltrindole and the κ-opioid receptor antagonist nor-binaltorphimine had no effect on the antinociceptive action of EM-1 [89]. In addition to this, μ_1-opioid receptor antagonist naloxonazine inhibits the antinociception induced by i.c.v administration of EM-2 more effectively than that of EM-1, whereas β-funaltrexamine inhibits both. These results suggest that antinociception induced by EM-1 is mediated by μ_2-opioid receptor, whereas EM-2 preferentially acts via μ_1-opioid receptor [42]. The μ_1-receptor mediates supraspinal analgesia, while μ_2-receptor associates with spinal analgesia and respiratory depression. Nevertheless, μ-opioid receptor subtype genes are not identified yet. To date, it has been proposed that the receptor subtypes found are a result of oligomerization of the opioid receptors or unique opioid receptors, which might have undergone discrete post-translational modifications [88, 90].

1.10. Function of endomorphins

Endomorphins have been postulated to represent the primary endogenous ligands for the μ-receptor [91]. They are considered to be partial agonists of μ-opioid receptors which are coupled to the superfamily of GPCR [46]. The G-proteins are heterotrimeric proteins consisting of G_α, G_β, G_γ subunits [18, 92]. Upon binding to the μ-opioid receptor endomorphins activate G-proteins inducing cellular responses including GDP dissociation by GTP on the G_α subunit, the reduction of cAMP formation and opening/closure of ion channels [22, 32 and 93]. The efficacies of endomorphins were found to be lower than that of DAMGO in [^{35}S]GTPγS binding assays, whereas the efficacy of endomorphins was measured similar to that of morphine [89, 94 and 95]. EM-1 partially antagonizes the DAMGO stimulation of [^{35}S]GTPγS binding which further confirms the partial agonist property of EM-1 [94]. As shown in various studies, observed efficacies confirmed that endomorphins are partial agonists [57, 79, 94 and 96]. The effects of EM-1 and EM-2 are comparable, only small differences were observed with respect to potency and efficacy in the case of the different cell lines used (SH-SY5Y human neuroblastoma, B82 fibroblast, Chinese hamster ovary (CHO) and C6 glioma cell lines) or rat and mouse brains/spinal cords [9]. In all studies, the effect of endomorphins was found to be reversible by μ-opioid antagonists [91, 92, 97, 98, 99 and 100] whereas δ- or κ-antagonists had no significant influence on the effects of these peptides in activating [^{35}S]GTPγS binding. The stimulation of the [^{35}S]GTPγS binding by endomorphins was diminished in homozygous knockout mice, while it was reduced to one-half of the wild-type levels in heterozygous mice [101, 102 and 103]. No stimulation of [^{35}S]GTPγS binding was observed by endomorphins with δ-opioid receptor transfected CHO cells in mouse [103]. These investigations suggest that μ-opioid receptor gene products play a key role in G-protein activation by endomorphins, and μ-opioid receptor densities could be rate-limiting steps in the G-protein activation by the μ-receptor reserve [9].

1.11. Radioactive isotopes as tracers

Isotope labeling is an essential tool for determining the fate of any given organic compound in a chemical or biochemical system. H, C, O, N and P are important elements in the biological systems. Since most of the organic compounds contain hydrogen and carbon, these isotopes are by far the most widely used in chemical and biological tracer studies. The first experiments with radioactive tracers were conducted in 1913 by György Hevesy and Friedrich A. Paneth. Hevesy was awarded the Nobel Prize in Chemistry in 1943 for his work on the use of isotopes as tracers in the study of chemical processes. Labeled compounds have significant importance in life sciences as tracers such as autoradiography, immunoassay, DNA-analysis and in direct tracing. In addition to this, radionuclides are being used in medical sciences as well. Currently, the medical imaging techniques, which use radionuclides (Transmission Tomography - CT, Emission Computed Tomography – SPECT, PET) are widely applied diagnostic methods in medicine. Radiotracers are also used for therapy such as internal or external sources.

The advantages of radioisotopes used as radiotracers are that they are chemically identical with the stable isotopes of the same element. The difference in the mass of the nucleus between the various isotopes does cause some change in the chemical and physical properties, but in most cases the isotope effect is rather small and difficult to detect. The radiotracers do not affect the system and can be used in nondestructive techniques and if the tracer is radiochemically pure, interference from other elements is of no concern [104].

Among the isotopes available, tritium-labeled ligands have proved to be essential for the *in vitro* characterization, autoradiographic localization and distribution studies of the receptors and biodegradation assays in opioid research. Tritium has become an important isotope for labeling ligands, having several advantages such as that the half-life of tritium (12.37 year) is relatively high and that tritium labeled compounds have a high specific radioactivity. The compounds with high specific radioactivity are useful for many biological investigations. Tritium labeling chemistry is often simple and labeling

can usually be accomplished very late in the overall synthesis, thus minimizing the radiochemical handling required.

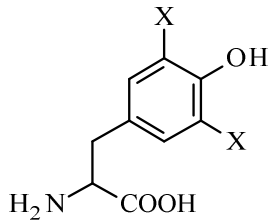
1.12. Tritium and tritium labeling methods of peptides

The existence of tritium was first predicted in the late 1920s by W. Russell, using his "spiral" periodic table, and then produced in 1934 from deuterium, another isotope of hydrogen, by E. Rutherford, working with M. Oliphant and P. Harteck. Rutherford was unable to isolate the tritium; the job was left to L. Alvarez and R. Cornog (1939) who correctly deduced that the substance was radioactive [105].

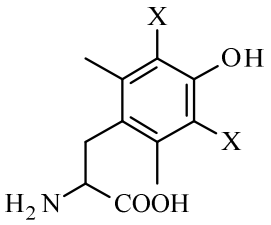
Tritium is a soft β-emitting radionuclide with low energy and low toxicity. The most advantageous property of tritiated compounds as tracers is the high stability of the tritium label under experimental conditions. Furthermore, its decay product, ^{3}He, is harmless. Hence, this hydrogen isotope is the safest radioactive nuclide in life sciences. Since having a high half-life (12.37 years), one tritium atom incorporated per molecule represents a specific radioactivity of 1.08 TBq/mmol (29.18 Ci/mmol). This value is appropriate for the study of neuropeptides in the nano- and subnanomolar ranges.

There are two basic methods for introducing tritium into organic molecules: the exchange method and the synthetic method [106]. The exchange reactions of $^{3}H/H$ isotope do not require separate synthetic steps. The disadvantage of this method is that the compounds are randomly labeled and high percent of impurities are formed during radiolytic side reactions. The main synthetic methods for tritium labeling of neuropeptides [107] include β-radiation induced isotope exchange reactions [108], catalytic isotope exchange [109] and chemical or enzymatic synthesis from precursor peptides or labeled amino acids, methylation of peptides with ^{3}H-methyl iodide [110] or reductive methylation using tritiated metal hydrides [111].

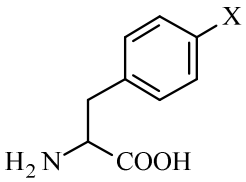
The appropriate precursor peptides can be obtained by postsynthetic modifications or by direct synthesis. Most of the precursor peptides for tritiation can be obtained by peptide synthesis using halogen containing saturatred or unsaturated amino acids. The amino acids most frequently used as precursors are 3',5'-diiodotyrosine and 3',5'-dibromotyrosine, *p*-iodophenylalanine or other *para*halogenated phenylalanine [112], 2',4'-diiodohistidine [113], 5',7'-dibromotryptophan [114], dehydroproline [115], dehydroleucine [116], dehydroisoleucine [117], 2-aminocyclopentenecarboxylicacid [118], 2-aminocyclohexenecarboxylicacid [119] and propargyl or allyl-glycine [120]. The schematic structures of these amino acids are represented in **Figure 2.**



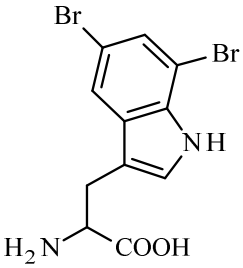
X= Br, 3',5'-dibromotyrosine
X= I., 3',5'-dibromotyrosine



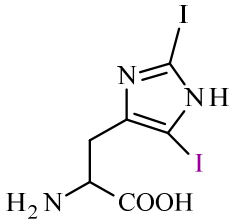
X= Br, 3',5'-dibromo-2',6'-dimethyltyrosine



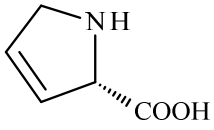
X= Cl, para-chlorophenylalanine
X= Br, para-bromophenylalanine
X=I, para-iodoophenylalanine



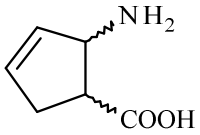
5'7'-dibromotryptophane



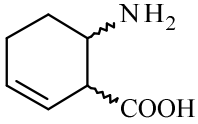
2',4'-diiodohistidine



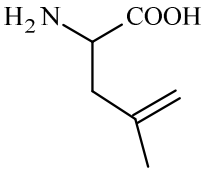
3, 4-dehydro-proline



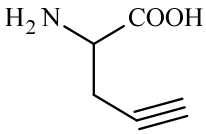
2-aminocyclopent-3-ene carboxylic acid (ΔAcpc)



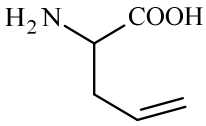
2-aminocyclohex-5-ene carboxylic acid (ΔAchc)



4,5-dehydroleucine



Propargyl glycine (Pra)



Allyl glycine

Figure 2. *Amino acid derivatives used for synthesis of precursor peptides for tritiation*

2. Aims and scope

Our research group has been working for years in the development of Dmt, Acpc, Achc, βMePhe and pFPhe containing endomorphin analogues in order to use as therapeutic opioid drugs. Introduction of alicyclic β-amino acids in the second position yielded proteolytically stable analogues with retained or enhanced biological activities based on the chirality of the incorporated amino acid [58]. By systematic incorporation of one unnatural amino acid in the sequence of endomorphins, analogues were synthesized and their pharmacological properties were reported previously [58, 59, 67, 68, 118, 119 and 121]. Designing the analogues with possible combination of these unnatural amino acids in the sequence could provide useful information in terms of pharmacological properties. By considering these facts, our aims were:

- ✓ To synthesize new endomorphin analogues with possible combination of Dmt^1, $Achc^2$, $\Delta Achc^2$/ $\Delta Acpc^2$, βPro^2, Hyp^2, $\beta MePhe^4$ and $pFPhe^4$ in order to obtain proteolytically stable and biologically active analogues.

- ✓ To investigate the affinities and selectivities of the newly synthesized analogues in radioligand receptor-binding studies.

- ✓ To test the functional properties of the highly potent analogues (selection based on receptor-binding assays results) in [^{35}S]GTPγS binding assay.

- ✓ To evaluate the enzymatic resistance of the highly potent analogues (selection based on receptor-binding and [^{35}S]GTPγS binding assay results) against proteolytic degrading enzymes, such as dipeptidyl peptidase IV carboxypeptidase Y, amino peptidases etc..

- ✓ To find out possible bioactive conformational structure(s) in relation with ligand-receptor interactions for μ-opioid receptor binding.

- ✓ To characterize blood-brain barrier permeabilities using tritiated EM-2 analogues.

- ✓ To test the analgesic properties of highly potent EM-2 analogues in *in vivo* chronic joint pain model.

- ✓ To perform structure-activity studies of highly potent EM-2 analogues to find out possible conformation(s) for μ-opioid receptor activity.

3. Materials and methods

3.1. Chemicals

The Boc-protected amino acids (with the exception of eβMePhe that was synthesized in our laboratory [68]) and 4-methylbenzhydralamine (MBHA) resin were purchased from Sigma-Aldrich Kft. (Budapest, Hungary) or from Bachem Feinchemikalen AG (Bubendorf, Switzerland). Boc-βPro was purchased from Polypeptide Group (Strasbourg, France). *cis*-(1*S*,2*R*)Achc was purchased form Peptides NeoMPS, USA in optical pure form. Racemic *cis*-(1*S*,2*R*)/(1*R*,2*S*) Achc was synthesized, purified and kindly provided by Prof. Ferenc Fülöp (University of Szeged, Hungary). Silica gel 60 F_{254} TLC plates were purchased from Merck (Darmstadt, Germany). Chiral TLC plates were purchased from Macherey-Nagel (Dürer, Germany). N,N-dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBt) were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Trifluoroacetic acid (TFA), dichloromethane (DCM), dimethylformamide (DMF), diisopropylethylamine (DIEA), ninhydrin, acetonitrile (ACN) and triethylamine (TEA) were purchased from Merck Kft. (Budapest, Hungary). Acetic acid (AcOH), methanol (MeOH), ethanol (EtOH) was delivered by Molar Kft. (Budapest, Hungary). All solvents were of analytical grade. Hydrogen fluoride was obtained form PRAXAIR N.V. (Oevel, Belgium). Guanosine-5'-O-(3-thio)triphosphate (GTPγS) was purchased from Amersham (GE Healthcare, United Kingdom). GF/B and GF/C filter papers were obtained from Whatman International Ltd. (Maidstone England, England). Tris-(hydroxymethyl)-aminomethane (Tris, free base), Bradford reagent, bovine serum albumin (BSA), sucrose, guanosine 5'-[β,γ-imido]-triphosphate trisodium salt, hydrochloric acid (HCl 37%) and anisole were purchased from Sigma-Aldrich Kft. (Budapest, Hungary). The dimethylsulfoxide (DMSO-d6) used for the ^{1}H-NMR analysis was purchased from Cambridge isotopes. [^{3}H]DAMGO (1.6 TBq/mmol, 43 Ci/mmol) [122], [^{3}H]Ile^{5,6}-deltorphin-2 (1.5 TBq/mmol, 39 Ci/mmol) [123] radioligands were prepared in our laboratory from the appropriate halogenated peptide derivatives. Radiolabeled

[^{35}S]GTPγS (>1000 Ci/mmol) was obtained from Institute of Isotopes Co., Ltd, (Budapest, Hungary).

3.2. Analytical methods

The analytical TLC (Thin Layer Chromatography) was performed on silica gel 60 F_{254} or on chiral TLC plates using the following solvent systems: (A) acetonitrile/methanol/water (4:1:1), (B) 1-butanol/acetic acid/water (4:1:1). The spots were visualized under UV light or with ninhydrin reagent. Analysis and separation of all compounds were performed with a complete HPLC system consisting of an L-7100 pump (Merck, KGaA, Darmstadt, Germany), a SIL-6B auto sampler (Shimadzu Co., Kyoto, Japan), a Shimadzu SCL-6B system controller, and a Merck l-7400 UV-vis detector, operating at 216 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 a Altima 218TP54 analytical C_{18} reverse phased column 218TP54 (250 mm × 4.6 mm, 5 μm, 300Å pore size) with a flow rate of 1mL/min. The compounds were separated on a semipreparative Vydac 218TP1010 column (250 mm × 10 mm, 12 μm) with a flow rate of 4 mL/min. The mobile phase was composed of 0.1 % (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile with gradient from 20 % up to 60 % ACN during 30 min. The identities of all peptides were confirmed by respective molecular weights and their purities were found to be >98%.The molecular weight of the peptides was determined by ESI-MS and HRMS. HRMS spectra were collected on a Waters QTOF Premier mass spectrometer (Waters Corporation, Milford, MA), using direct infusion to the nanoelectrospray source. Samples were dissolved in 0.1% (v/v) formic acid/20% (v/v) acetonitrile/water solvent to a final concentration of ~0.01 mg/mL. The mass spectra were collected in the 50-990 Da range; the spectrometer was calibrated by using the MS/MS fragments of a 100 fmol/μL [Glu^{1}]-Fibrinopeptide B solution.

For binding experiments, incubation mixtures were filtered using a M24R Brandel cell harvester (Gaithersburg, MD). Filter-bound radioactivities were detected and measured in a TRI-CARB 2100TR liquid scintillation analyzer (Packard) using biodegradable Optiphase Supermix cocktail developed by PerkinElmer, USA. Inhibitory constants (K_i, nM) were calculated from the competition experiments by using nonlinear least-squares curve fitting and the Cheng-Prusoff equation, [23] potency (ED_{50}) and efficacy (E_{max}) values were calculated with GraphPad Prism software (version 4.0, San Diego, CA).

3.3. Peptide synthesis, purification and determination of configuration of the incorporated alicyclic β-amino acids

The synthesis of the peptide analogues was performed by manual solid phase technique using N-Boc-protected amino acids and MBHA resin on 0.25 mmol scale. Boc-protected amino acids were added to pre-swelled resin (in 3.0 ml of DCM for 60 min) in 2:1 (mmol) ratio. Coupling agents (HOBt and DCC) were applied in 1:1 (mmol) ratio regarding to amino acid. Unreacted amino acids and coupling agents were removed by washing the resin with dichloromethane (DCM) and ethanol (each 2.5 ml). The protective groups were removed with a 2.5 ml × 2 (5 and 20 min) solution containing 50% TFA, 48% DCM and 2% anisole, followed by neutralization with 2.5 ml × 2 (2 min each) solution consisting of 10% diisopropyl-ethylamine (DIEA) in DCM. Each coupling was monitored by the Kaiser test [124]. The cleavage of the peptides from the resin was accomplished with anhydrous HF (10 mL/g resin) in presence of anisole (1 mL/g resin) and dimethylsulfide (1 mL/g resin) at 0^oC for 60 min. After evaporation of the HF, the resin was washed with diethyl ether to remove the scavengers, and the peptide was diluted in 30% (v/v) aqueous acetic acid. Crude peptides were obtained in solid form after lyophilization of the diluted extract (yields 70-80%). The diastereomers were separated using semipreparative RP-HPLC on a Vydac 218TP1010 C_{18}column. Purities were found to be over 98% as assessed by analytical RP-HPLC. Erythro (2*S*,3*S* and 2*R*,3*R*)βMePhe containing peptides were purified by silica gel column chromatography due to the same retention time of the diastereomers with RP-HPLC. The eluent used for

compounds **2**-**5**, **11**, **12** was DCM/MeOH 80:20 (v/v) and for compounds **13** and **14** the eluent was EtOAC/AcOH 70:30 (v/v). These peptides were further purified by RP-HPLC to obtain maximal purity (≥98%).

The configuration of the incorporated Acpc/Achc was determined after GITC derivatization of the acidic hydrolysates of the peptides (for acidic hydrolysis, 1 mg of peptide, 1 mL of 6M HCl, 24 h, 110^{o}C) followed by analytical HPLC analysis. The retention times of the derivatized alicyclic β-amino acid were compared with those of the derivatized β-amino acid standards [125]. The configuration of βMePhe was determined with chiral TLC after acidic hydrolysates of the peptides using solvent system A. These R_f values were compared with those of standard optically pure βMePhe isomers [126].

3.4. Tritium labeling of peptides

The conditions used in tritium labeling were optimized in inactive circumstances, using hydrogen gas in order to determine proper reaction conditions such as catalyst, reaction time and solvent. Labeling of peptides with tritium was performed in our in-house designed vacuum apparatus [127] under a fume cupboard. About 2 μmol of pure peptide was dissolved in 1 mL of DMF and 10 mg of catalyst (PdO/BaSO4) was added. In most cases, an excess of TEA was added to neutralize HI formed during the reaction. The reaction vessel was connected to the tritiation manifold frozen with liquid nitrogen and the air was removed by vacuum. Tritium gas 555GBq (15 Ci) was liberated by heating (300^{o}C) from uranium tritide and was introduced into the reaction vessel. The reaction mixture was stirred by a magnetic stirrer at room temperature for 1-2 hours. The reaction was terminated by freezing the solution and absorbing the unreacted tritium on pyrophoric uranium. The catalyst was filtered off using Whatmann GF/C glass-fiber filter paper. Labile tritium was removed by repeated evaporation with ethanol/water 1:1 mixture. The crude reaction mixture was analyzed by radio-HPLC. A TRI-CARB 2100TR liquid scintillation analyzer (Packard) was used to determine the total radioactivity of crude radioligand with a biodegradable Optiphase Supermix cocktail

developed by Perkin-Elmer (USA). The specific activity was determined by means of calibration curve prepared with the appropriate inactive peptide standard. After the determination of specific activity, radiolabeled peptides were stored in liquid nitrogen, showing good stability during long storage (more than 1 year).

3.5. Animals

Inbred Wistar rats (male, 250–300 g body weight) were housed in the local animal house of the Biological Research Center (BRC, Szeged, Hungary). Rats were kept in-groups of four, allowed free access to food and water and maintained on a 12:12-h light/dark cycle until the time of sacrifice. Animals were treated according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

3.6. Rat brain membrane preparation

Whole brains minus cerebella were dissected and crude membrane fractions were prepared as published earlier [21]. Briefly, the brains were homogenized in a 30 volumes (v/w) of ice-cold 50 mM Tris-HCl (pH = 7.4) buffer with a Teflon-glass Braun-homogenizer at 1500 rpm. The homogenate was centrifuged at 20,000 *x g* for 25 minutes and the supernatant was discarded. The resulting pellet was resuspended in the same volume of cold buffer followed by incubation at 37^{o}C for 30 minutes to remove endogenous ligands. Centrifugation was then repeated. The final pellets were taken up in 5 volumes of cold 50 mM Tris-HCl (pH = 7.4) buffer containing 0.32 M sucrose and stored at -80°C. Membranes were thawed before use, diluted with the working buffer and centrifuged at 20,000 x g for 25 min at 4^{o}C to remove sucrose. The resulting pellet was homogenized with a Dounce homogenator in 60 volumes of buffer to yield protein concentrations of about 0.3 mg/ml. The protein content of the membrane preparation was determined by the method of Bradford using bovine serum albumin as standard [128].

3.7. Radioligand-binding assay

In competition binding experiments the conditions were optimized and varied, based on the radioligand applied. Competition binding experiments were performed by incubating rat brain membranes (0.2-0.5 mg protein/tube) with [^{3}H]DAMGO (1 nM, 25^{o}C, 1 h, GF/C filter, glass tubes) or [^{3}H]Ile^{5,6}-deltorphin-2 (2 nM, 35^{o}C, 45 min., GF/B filter, plastic tubes) and with increasing concentrations (10^{-10} – 10^{-5}) of unlabeled endomorphin analogues. The incubation mixtures were made up to a final volume of 1 mL with 50 mM Tris-Cl buffer (pH = 7.4) and samples were incubated in a shaking waterbath at the appropriate temperature. Nonspecific binding was measured with 10 μM naloxone and subtracted from the total binding to determine specific binding. Incubation was initiated by the addition of the membrane preparation and stopped by the rapid filtration over Whatman GF/C or GF/B glass fiber filters, using a Brandel Cell Harvester (Gaithersburg, MD, USA). Vials were washed with 3×5 mL of ice–cold 50 mM Tris-HCl buffer (pH = 7.4), dried at room temperature for 15-20 min. The filter bound radioactivities were measured in an Optiphase Supermix scintillation cocktail using a TRI-CARB 2100TR liquid scintillation counter (Canberra-Packard, Perkin-Elmer Life Sciences 549 Albany Street, Boston MA 02118). The inhibitory constants, K_i values were calculated from the displacement curves by using non-linear least-square curve fitting and the Cheng-Prusoff equation with GraphPad Prism software (version 4.0, San Diego, CA). The data were expressed as means ± SEM of at least three independent measurements, each performed in duplicate.

3.8. Ligand-stimulated [^{35}S]GTPγS functional binding assay

Rat brain membranes (10-15 μg protein/tube) were incubated with 0.05 nM [^{35}S]GTPγS and 10^{-10}–10^{-5} M concentrations of unlabeled ligands in the presence of 30 μM GDP in Tris-EGTA buffer (50 mM Tris-HCl, 100 mM NaCl, 3 mM $MgCl_2$ and 1 mM EGTA, pH = 7.4) at 30^{o}C for 60 min in absence or presence of naloxone (10^{-5} M). Basal activity was determined by subtracting the nonspecific binding (measured in the presence of 10 μM unlabeled GTPγS) from the total binding (measured in the absence of tested compounds). The incubation was started by the addition of [^{35}S]GTPγS and was terminated by vacuum filtration through Whatman GF/B glass fiber filters with a Brandel Cell Harvester. Filters were washed with 3×5 mL of ice–cold 50 mM Tris-HCl buffer (pH=7.4) and then dried at room temperature for 15-20 min. The radioactivity was counted in an Optiphase Supermix cocktail using a TRI-CARB 2100TR liquid scintillation counter (Canberra-Packard, Perkin-Elmer Life Sciences). Stimulation is given as percentage of the basal activity. The potency (EC_{50} the concentration of the ligand required to elicit the half-maximal effect), and efficacy (E_{max}, the maximal stimulation of a drug over basal activity) were calculated by using Sigmoidal Dose-Response curve fitting option of GraphPad Prism software (version 4.0, San Diego, CA). The data were expressed as means ± SEM of at least three independent measurements, each performed in triplicate.

3.9. Determination of the half-lives

20 μL of 1mM peptide stock solution in 50 mM Tris-HCl buffer (pH = 7.4) was added to 180 μL of the rat brain homogenate (protein concentration: 5.0 mg/mL) and the mixtures were incubated at 37^{o}C. Aliquots of 20 μL were withdrawn from the incubation mixtures and immediately 25 μL of 0.1 mM aqueous HCl solution was added to halt the degradation. After centrifugation (11340 g, 5 min, 25^{o}C) of the incubation mixtures, 10 μL of obtained supernatant was analyzed by RP-HPLC. The rate constants (k) of

degradation were determined by least square linear regression analysis of logarithmic tetrapeptide peak areas [(ln(A/Ao)] vs time courses, with minimum of five time points. The obtained rate constants were used to establish the degradation half-lives ($t_{1/2}$) as ln2/*k*.

3.10. Study of BBB of some endomorphin analogues

a. Determination of n-octanol/water partition coefficient

The values of n-octanol/water partition coefficients (Log *P*), characterizing the hydrophobicity of the synthesized peptides, were expressed as the ratio of peptide concentration found in the octanol phase to that found in the aqueous phase. Equal volumes of n-octanol and 0.05 M HEPES buffer in 0.1 M NaCl, pH = 7.4, were mixed and allowed to equilibrate for 12 h. The layers were then separated and stored at 4°C. At testing, 0.5 mg of peptides were dissolved in 500 µl of 0.05 M HEPES buffer, then 500 µl of n-octanol was added and mixtures were vortexed for 2 min. Samples were centrifuged at 4000 rpm for 1 min. After separation, octanol phase was lyophilized and reconstituted in acetonitrile/water containing 0.1% TFA. Octanol and aqueous phases were used to quantify peptide content by RP-HPLC (Altima C_{18}, 5 µm, 4.6 mm × 250 mm) using linear gradient of 5-60% solvent B over 25 min. All n-octanol/buffer distribution studies were performed in triplicate.

b. Measurement of the permeability of the peptides

Transwell filters containing endothelial cells were removed from the plates containing the glial cultures. Filters were washed with Ringer-HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 5 mM HEPES, 2.8 mM D-glucose, pH = 7.4). The peptides were applied in a final concentration of 0.01-1000 µM and 0.381-0.775 µCi/ml (depending on the specific activity of the radiolabeled

peptide) to the apical (luminal) side of the endothelial monolayer in Ringer-HEPES. The basolateral (abluminal) side of the filters was loaded with Ringer-HEPES. Samples were taken from the abluminal side after 1 h incubation at 37°C with gentle shaking. All experiments were performed in triplicate.

The radioactivity was measured in a TRICARB 2100TR liquid scintillation analyzer (Packard) using toluol-Triton-x cocktail containing Liquidflour.
For the measurement of permeability coefficient, the peptides were applied to the luminal side in a concentration of 0.01 μM and 0.381-0.775 μCi/ml. After 1 h samples were taken from the abluminal side.

The permeability coefficient was calculated using the following formula:

$$P = \frac{dQ}{dT \cdot A \cdot C_0}$$

(dQ: transported amount, dT: incubation time, A: surface of filter, C_0: initial concentration in the luminal compartment).

The calculated P_{total} values were compared to the P_{filter} of the empty filter and the real P_e value for the endothelial monolayer was calculated using the following formula:

$$\frac{1}{P_e} = \frac{1}{P_{total}} - \frac{1}{P_{filter}}$$

The permeability of the peptides was compared to the permeability of sodium fluorescein (SF, MW = 376 Da) and Evans blue labeled albumin (EBA, MW = 67 kDa). The luminal side was loaded with Ringer-HEPES containing 10 μg/ml SF, 170 μg/ml Evans blue and 10 mg/ml BSA. The cells were incubated at 37ºC for 1 h with gentle shaking, and samples were collected from the abluminal side. Concentration of SF and EBA was measured using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm for SF, and 584/680 nm excitation/emission wavelengths for EBA. The permeability coefficient (P_e) was calculated similarly.

4. Results

4.1. Incorporation of unnatural amino acids into endomorphins

In general, endomorphins are proteolytically less stable, although having the longest half-lives among the known opioid peptides [31, 50, 51 and 76]. It is essential to enhance the CNS entry of endomorphins and their resistance to enzymatic degradation for considering them as valuable therapeutic drugs. In order to increase the proteolytic stability while retaining or enhancing the biological activity of endomorphins, we introduced unnatural amino acids such as 2, 6-dimethyltyrosine[1] (Dmt), (2*S*,4*R*)-hydroxyproline[2] (Hyp), (S)-β-proline[2] (βPro), *cis*-2-aminocyclohexanecarboxylic acid[2] (*cis*-Achc), *cis*-2-aminocyclopentenecarboxylic acid[2] (*cis*-ΔAcpc), *cis*-2-aminocyclohexenecarboxylic acid[2] (*cis*-ΔAchc), erythro-β-methylphyenylalanine[4] (eβMePhe) and para-fluoro-phenylalanine[4] (pFPhe) into the sequence of endomorphins. Incorporated unnatural amino acids structures are presented in **Figure 3A** and **3B**.

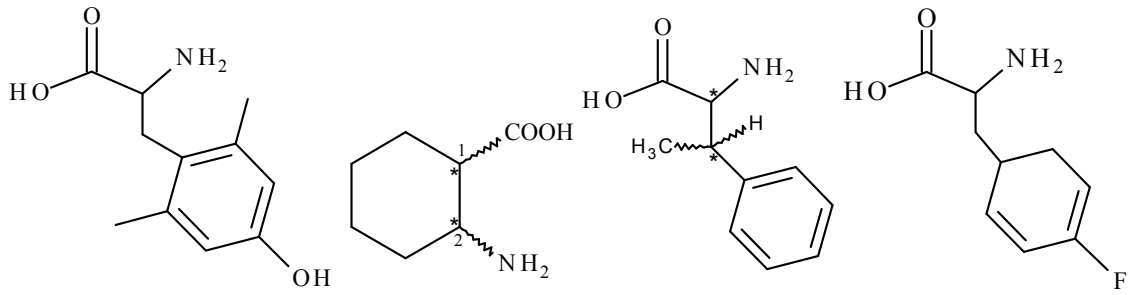
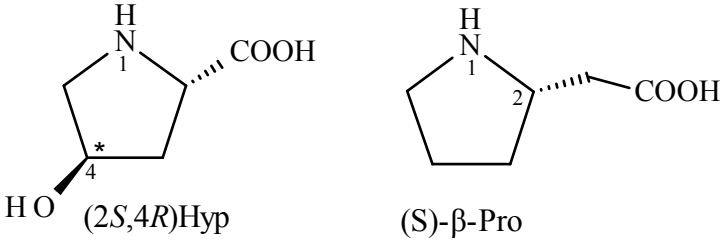
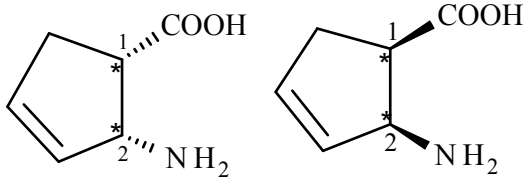


Figure 3A. *Chemical structures of unnatural amino acids incorporated in analogues* ***2-8*** *and* ***10-16***.



2-aminocyclopentenecarboxylic acid



2-aminocyclohexenecarboxylic acid

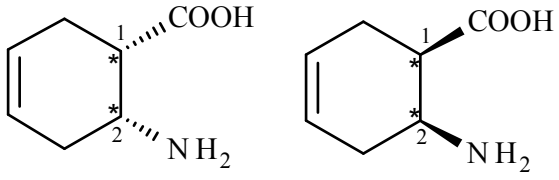


Figure 3B. *Chemical structures of unnatural amino acids incorporated in second position of EM-2 (analogues 17-21).*

4.2. Synthesis of novel endomorphin analogues

All the peptide analogues were synthesized by manual solid phase peptide synthesis method using Boc-protected amino acids. Racemic saturated or unsaturated alicylic β-amino acids were incorporated with combination of Dmt or pFPhe to obtain diasteromeric peptide analogues. The analytical properties of the new analogues are reported in **Table 3**. Erythro-βMePhe was used to obtain respective peptide diastereomers. Optically pure *cis*-(1*S*,2*R*)Achc was used for the synthesis of peptides containing βMePhe. Racemic unsaturated alicylic β-amino acids (*cis*-ΔAcpc and *cis*-ΔAchc) were used in respective endomorphin derivatives. The obtained crude peptides indicated that the ratio of the diastereomeric peptides was nearly 1:1. Crude peptides were purified by semi-preparative RP-HPLC. The configuration of cis-alicyclic-β-amino acids was determined after GITC derivatization of the acidic hydrolysates of the peptides followed by analytical RP-HPLC analysis. The retention times of the derivatized alicyclic β-amino acid were compared with those of the derivatized β-amino acid standard [125]. Configuration of βMePhe residues in the peptide analogues was determined by chiral TLC analysis of the acidic hydrolysates of the peptides by comparing R_f values with those of standard optically pure βMePhe isomers [126]. The (2*S*,3*S*)βMePhe isomers had higher R_f values than those of the corresponding (2*R*,3*R*)βMePhe isomers in an eluent mixture of acetonitrile-methanol-water (4:1:1). The molecular weight for all analogues was determined by ESI-MS or High Resolution Mass Spectrometry (HRMS). The identity of all peptides was confirmed and their purities were found to be over 98% as assessed by analytical RP-HPLC.

Table 3. Analytical properties (RP-HPLC, TLC and HMRS/ESI-MS data) of endomorphins and their derivatives

no.	Peptide	TLC[a]		HPLC[b] (k')	monoisotopoic mass cal.	measured HRMS
		R_f (A)	R_f (B)			
1	Tyr-Pro-Trp-Phe-NH_2	0.61	0.70	3.84	610.27	611.37[c]
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	0.55	0.83	6.09	652.3374	652.3344
3	Tyr-(1*S*,2*R*)Achc-Trp-(2*R*,3*R*)βMePhe-NH_2	0.59	0.85	6.04	652.3374	652.3419
4	Dmt-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	0.52	0.84	6.47	680.3686	680.3623
5	Dmt-(1*S*,2*R*)Achc-Trp-(2*R*,3*R*)βMePhe-NH_2	0.59	0.84	6.39	688.3686	688.3796
6	Tyr-(1*S*,2*R*)Achc-Trp-pFPhe-NH_2	0.58	0.81	6.18	656.3123	656.3104
7	Tyr-(1*R*,2*S*)Achc-Trp-pFPhe-NH_2	0.70	0.76	4.69	656. 3123	656.3215
8	Dmt-(1*S*,2*R*)Achc-Trp-pFPhe-NH_2	0.57	0.83	6.58	684.3435	684.3399
9	Tyr-Pro-Phe-Phe-NH_2	0.56	0.68	3.57	571.26	572.29[c]
10	Tyr-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	0.58	0.81	6.10	613.3265	613.3291
11	Tyr-(1*S*,2*R*)Achc-Phe-(2*R*,3*R*)βMePhe-NH_2	0.59	0.82	6.17	613.3265	613.3303
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	0.51	0.83	6.58	641.3577	641.3605
13	Dmt-(1*S*,2*R*)Achc-Phe-(2*R*,3*R*)βMePhe-NH_2	0.59	0.85	6.48	641.3577	641.3591
14	Tyr-(1*S*,2*R*)Achc-Phe-pFPhe-NH_2	0.56	0.80	6.14	617.3014	617.3071
15	Tyr-(1*R*,2*S*)Achc-Phe-pFPhe-NH_2	0.68	0.75	4.67	617.3014	617.3107
16	Dmt-(1*S*,2*R*)Achc-Phe-pFPhe-NH_2	0.52	0.83	6.82	645.3326	645.3371
17	Tyr-Hyp-Phe-Phe-NH_2	0.58	0.91	1.49	587	588.25[c]
18	Tyr-βPro-Phe-Phe-NH_2	0.43	0.67	1.97	585	586.28[c]
19	Tyr-(1*S*,2*R*)ΔAcpc-Phe-Phe-NH_2	0.56	0.82	2.51	583	584.25[c]
20	Tyr-(1*R*,2*S*)ΔAcpc-Phe-Phe-NH_2	0.67	0.71	1.71	583	584.31[c]
21	Tyr-(1*S*,2*R*)ΔAchc-Phe-Phe-NH_2	0.50	0.76	2.65	597	598.29[c]
22	Tyr-(1*R*,2*S*)ΔAchc-Phe-Phe-NH_2	0.68	0.67	1.99	597	598.22[c]

[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 Altima HP C_{18} (25 × 0.46 cm, d_p = 5 μm) column. The gradient was from 20 % up to 60 % ACN during 30 min., at a flow rate of 1 mL/min., λ = 216 nm. [c]Measured mass with ESI-MS.

4.3. Receptor binding affinities and selectivities of new endomorphin analogues

The binding affinities and selectivities of the newly synthesized endomorphin analogues were evaluated by radioligand binding assays using rat brain membrane preparations. The opioid prototype, enkephalin derivative [^{3}H]DAMGO, a highly selective μ-opioid receptor ligand, was used to evaluate μ-opioid receptor binding affinities. The synthetic [^{3}H]Ile^{5,6}-deltorphin-2 peptide ligand was used for assessment of δ-receptor affinities. All the new compounds competed with the radiolabeled μ- and δ-receptor ligands in a concentration dependent manner for the receptor binding sites. The inhibitory constants (K_i) and selectivities (K_i^{δ}/K_i^{μ}) of the 22 new analogues over the values of the parent ligands are listed in Table 4. EM-1 and -2 were also evaluated for comparison and the values obtained were consistent with literature data [38, 58 and 68]. The representative curves of μ and δ-opioid receptors of some of highly potent analogues are shown in **Figure 4**. The analysis of the binding results revealed that the co-application of (1*S*,2*R*)Achc^2 and (2*S*,3*S*)βMePhe^4 in both endomorphins resulted comparably potent analogues (compounds **2** and **10**) compared to the native peptides. These binding potencies were further enhanced by the combined substitution of Dmt^1, (1*S*,2*R*)Achc^2 and (2*S*,3*S*)βMePhe^4 accomplished in compounds **4** and **12**. The results confirmed that single or combined use of Dmt^1 increases the affinity for μ-opioid receptor but, at the same time, decreases selectivity of the ligands. Analogues containing the corresponding (2*R*,3*R*)βMePhe^4 residue exhibited lower affinities than those including the other isomer (2*S*,3*S*)βMePhe^4, however, replacement of Tyr^1 with Dmt could enhance the binding potency of the ligands (compounds **5** and **13**). Co-substitution with the halogenated pFPhe^4 and Achc^2 resulted in ligands with different potencies depending on the chirality of the alicyclic β-amino acids (compounds **6-8**, **14-16**). In comparison with compounds **5** and **13**, it is interesting to note that compound **16** showed higher potency than its corresponding EM-2 analogue therefore, it can be assumed that only minor differences in structures (Trp/Phe) may be responsible for the observed changes in ligand binding. Furthermore, it is also interesting to mention that pFPhe^4 could compensate the detrimental effects of (1*R*,2*S*)Achc^2 incorporation as it was

demonstrated for compound **15** (K_i=2.8 nM) and other endomorphin derivatives. Endomorphins containing (1*R*,2*S*)Achc[2] displayed less μ-opioid receptor affinity (K_i = 741 nM, 1984 nM for EM-1, and -2 respectively) compared to parent compounds [58]. Among the pFPhe[4] containing analogues, compound **16** displayed the highest μ-opioid receptor affinity with relatively high selectivity. Incorporation of Hyp[2] (compound **17**) resulted in decreased affinity to μ-opioid receptor (K_i=44 nM), in contrast to affinity values measured with opioid selective antagonist naloxone in crude rat brain membrane [129]. Insertion of (*S*)-βPro[2] resulted in lower affinity (K_i=27 nM) compared to that of parent EM-2. Unlike EM-2, βPro[2]containing EM-1 exhibited higher affinity to μ-opioid receptor in rat brain homogenate [130]. Compound **19** and **21**, bearing *cis*-(1*S*,2*R*)ΔAcpc[2]/*cis*-(1*S*,2*R*)ΔAchc[2] displayed higher affinities compared to other isomer (1*R*,2*S*). The observed propensity of binding potencies for unsaturated alicyclic β-amino acids analogues followed the results obtained for respective saturated alicyclic β-amino acid analogues [58]. Compound **19** exhibited equal affinity (K_i=1.3 nM) and high selectivity for μ-opioid receptor relative to the parent EM-2. The rank order of potency measured against [^{3}H]DAMGO was as follows compounds 16<12<10<4<19<9<14<1<15<6<5<2<13<8<18<11<3<20<7<20<22. Each ligand showed moderate to low binding affinities for δ-opioid receptors, indicating that these modifications provide μ-opioid receptor ligands.

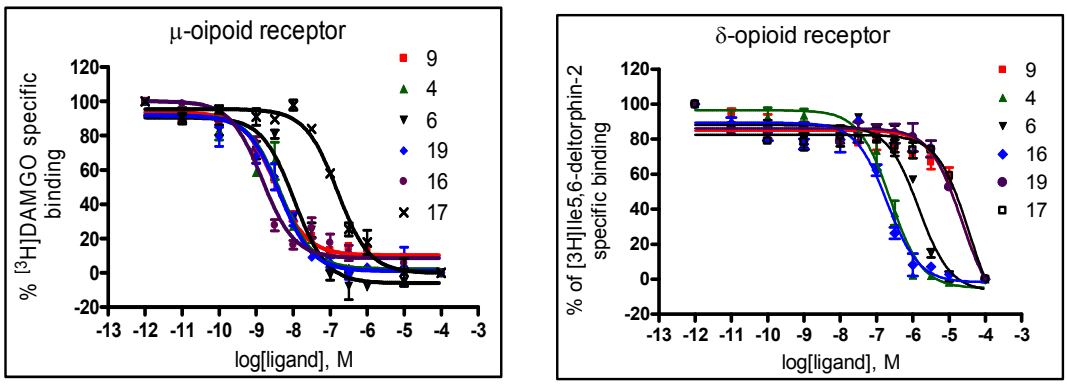


Figure 4. *Representative curves of competitive receptor binding of novel endomorphin analogues at μ- and δ-opioid receptor sites. Points represent the means ± SEM of at least three different experiments, each performed in duplicate.*

Table 4. Summary of inhibitory constants (K_i) and selectivity of endomorphins and their analogues measured on rat brain membrane preparation

no.	peptide	inhibitory constants		selectivity
		K_i^{μ} (nM)[a]	K_i^{δ} (nM)[b]	K_i^{δ}/K_i^{μ}
1	Tyr-Pro-Trp-Phe-NH_2	1.6 ± 0.3	4169 ± 881	2605
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	4.2 ± 0.9	1444 ±182	343
3	Tyr-(1*S*,2*R*)Achc-Trp-(2*R*,3*R*)βMePhe-NH_2	34.6 ± 3.5	3364 ± 1093	97
4	Dmt-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	0.93 ± 0.07	123 ± 17	132
5	Dmt-(1*S*,2*R*)Achc-Trp-(2*R*,3*R*)βMePhe-NH_2	4.11 ± 0.7	287 ± 55	69
6	Tyr-(1*S*,2*R*)Achc-Trp-pFPhe-NH_2	3.2 ± 0.5	571 ± 92	178
7	Tyr-(1*R*,2*S*)Achc-Trp-pFPhe-NH_2	143.1 ± 5.9	7823 ± 1039	54
8	Dmt-(1*S*,2*R*)Achc-Trp-pFPhe-NH_2	11.1 ± 2.0	2636 ± 670	237
9	Tyr-Pro-Phe-Phe-NH_2	1.35 ± 0.2	8771 ± 1316	6497
10	Tyr-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	0.82 ± 0.2	661 ± 43	816
11	Tyr-(1*S*,2*R*)Achc-Phe-(2*R*,3*R*)βMePhe-NH_2	33.9 ± 6.0	1268 ± 33	37
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	0.47 ± 0.06	142 ± 8	302
13	Dmt-(1*S*,2*R*)Achc-Phe-(2*R*,3*R*)βMePhe-NH_2	9.7 ± 1.3	198 ± 48	20
14	Tyr-(1*S*,2*R*)Achc-Phe-pFPhe-NH_2	1.5 ± 0.3	366 ± 61	244
15	Tyr-(1*R*,2*S*)Achc-Phe-pFPhe-NH_2	2.8 ± 0.5	689 ± 98	246
16	Dmt-(1*S*,2*R*)Achc-Phe-pFPhe-NH_2	0.13 ± 0.02	96 ± 9	738
17	Tyr-Hyp-Phe-Phe-NH_2	44 ± 3.4	> 10000	-
18	Tyr-βPro-Phe-Phe-NH_2	27 ± 2.6	> 10000	-
19	Tyr-(1*S*,2*R*)ΔAcpc-Phe-Phe-NH_2	1.3 ± 0.2	9935 ± 1218	7642
20	Tyr-(1*R*,2*S*)ΔAcpc-Phe-Phe-NH_2	2158 ± 213	> 10000	-
21	Tyr-(1*S*,2*R*)ΔAchc-Phe-Phe-NH_2	2.6 ± 0.2	1028 ± 121	395
22	Tyr-(1*R*,2*S*)ΔAchc-Phe-Phe-NH_2	2299 ± 372	> 10000	-

[a][^{3}H]DAMGO (K_d= 0.5 nM) was used as radioligand for the μ-opioid receptor. [b][^{3}H]Ile^{5,6}deltorphin-2 (K_d= 2.0 nM) was used as a radioligand for the δ-opioid receptor. K_i values were calculated according to the Cheng-Prusoff equation: $K_i = EC_{50}/(1+[ligand]/K_d)$. Data are expressed as means ± SEM, $n \geq 3$.

4.4. [^{35}S]GTPγS functional assay of the new endomorphin analogues

On the basis of the heterologous displacement binding results, the most potent analogues were selected for [^{35}S]GTPγS functional assays. The results of the ligand-stimulated [^{35}S]GTPγS functional assays are summarized in Table 5. From the pool of ligands, compounds **2**, **4**, **6**, **8**, **10**, **12**, **14** and **16**, and the parent peptides were undertaken to overall functional characterization and comparison. Representative curves of G-protein stimulation of some of the ligands were shown **Figure 5**. To confirm opioid receptor functionality, the newly synthesized ligands were also assayed in the presence of the opioid selective antagonist naloxone. Potency (EC_{50}) and efficacy (E_{max}) values were compared with those of the μ-receptor full agonist DAMGO (compound **0**). Dose-dependent increases were observed for the selected compounds in [^{35}S]GTPγS binding. Both parent ligands exhibited comparable potencies, but lower efficacies (E_{max}=149% for EM-1 and E_{max}=163% for EM-2) could be observed as compared to DAMGO, confirming that endomorphins are partial agonists, as reported earlier [94, 96, 131 and 132]. Compounds **6**, **10** and **16** displayed the highest efficacies (E_{max}=174%, E_{max}=173%, E_{max}=176%, respectively), acting as full agonists. Additionally, compound **16** showed four times higher potency (EC_{50}=51 nM) than that of EM-2 (EC_{50}=212 nM). Compounds **2**, **4**, **8**, **12** and **14** revealed slightly less efficacies than that of DAMGO, which indicate partial agonist or full agonist properties. Interestingly, compound **12** displayed the highest potency (EC_{50}=17 nM) among all analogues. All analogues moderately stimulated G-protein activation. Compound **17** had moderate efficacy (E_{max}=148%), suggesting that it is a weak partial agonist. The lowest efficacy (E_{max}=116%) was observed for compound **18** among all new analogues, suggesting it is a moderate agonist. Compound **19** displayed comparable efficacy (E_{max}=170%) with μ-receptor selective agonist DAMGO (E_{max}=173%), proposing a full agonist property. Compound **21** showed efficacy (E_{max}=162%) comparable to the parent EM-2, acting as a partial agonist. In the case of EM-1, insertion of βPro^2 yielded a peptide with an increased efficacy, acting as an agonist [87]. Interestingly, compounds **4**, **12** and **16** stimulated G-protein binding even in the presence of 10^{-5} M naloxone (data not shown) therefore, we hypothesized that these ligands may bind to other G-protein coupled receptors or to non-opioid binding sites. The

rank order of efficacy (E_{max}) measured by [^{35}S]GTPγS functional assay was: compounds 0>16>6>10>19>14>8=9>2=4=21>12>1>17>18.

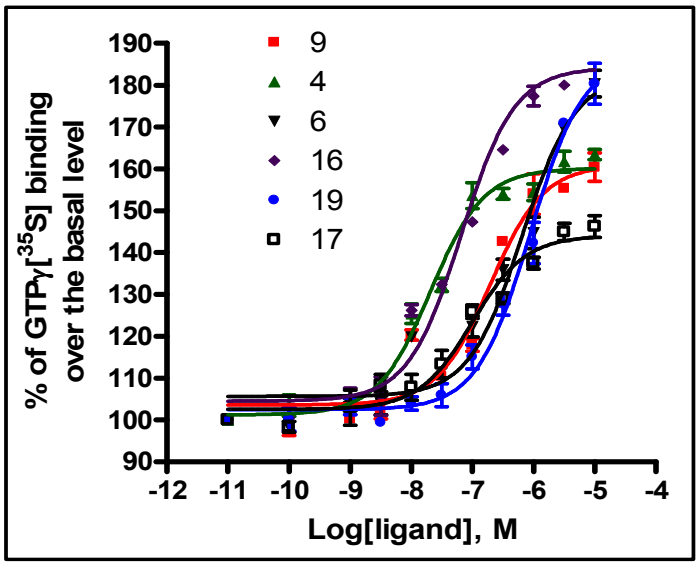


Figure 5. *Stimulation of G-proteins by novel endomorphin analogues in rat brain membranes. Basal activity (basal = 'total binding' – 'nonspecific binding') is taken as 100%. Nonspecific binding is determined in the presence of 10 μM unlabeled GTPγS. Points represent the means ± SEM of at least three independent measurements, each performed in triplicate.*

Table 5. Summary of [^{35}S]GTPγS functional assays with selected endomorphin analogues measured in a rat brain membrane preparation

no.	peptide	EC_{50} (nM)	E_{max} (%)
0	DAMGO	214 ± 39	178 ± 5
1	Tyr-Pro-Trp-Phe-NH_2	166 ± 27	149 ± 4 ^{ns}
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	264 ± 8	162 ± 2 ^{ns}
4	Dmt-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	22.1 ± 3	162 ± 9 ^{ns}
6	Tyr-(1*S*,2*R*)Achc-Trp-pFPhe-NH_2	380 ± 38	174 ± 9 ^{ns}
8	Dmt-(1*S*,2*R*)Achc-Trp-pFPhe-NH_2	8.1 ± 3	163 ± 13 ^{ns}
9	Tyr-Pro-Phe-Phe-NH_2	212 ± 6	163 ± 6 ^{ns}
10	Tyr-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	273 ± 31	173 ± 6 ^{ns}
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	17 ± 5	159 ± 4 ^{ns}
14	Tyr-(1*S*,2*R*)Achc-Phe-pFPhe-NH_2	92 ± 8	168 ± 6 ^{ns}
16	Dmt-(1*S*,2*R*)Achc-Phe-pFPhe-NH_2	51 ± 8	176 ± 7 ^{ns}
17	Tyr-Hyp-Phe-Phe-NH_2	66 ± 38	148 ± 9 ^{ns}
18	Tyr-βPro-Phe-Phe-NH_2	845 ± 109	116 ± 2 ***
19	Tyr-(1*S*,2*R*)ΔAcpc-Phe-Phe-NH_2	345 ± 12	170 ± 12 ^{ns}
21	Tyr-(1*S*,2*R*)ΔAchc-Phe-Phe-NH_2	1024 ± 3	162 ± 6 ^{ns}

Sigmoid dose-response curves of the listed peptides were determined as described in the Methods section. EC_{50} and E_{max} values were calculated by using the sigmoid dose-response fitting option of the GraphPad Prism software. The concentration of naloxone used to block ligand binding and subsequent G-protein activation was set at 10^{-5} M. Data are expressed as the % stimulation of the basal activities in the absence of peptides, which was defined as 100%. Data are means ± SEM, $n \geq 3$, each performed in triplicate. *** = $P<0.001$ as assessed by ANOVA and Bonferroni's post hoc test (compared to DAMGO).

4.5. Enzymatic degradation

The stability studies of the most potent analogues towards enzymatic degradation were performed in rat brain homogenate. Compound **17** displayed a 3 fold higher half-life (17 min) relative to the parent compound EM-2. Insertion of β-homoproline (compound **18**) lead to an increase of 6 folds in stability, compared to the parent compound EM-2. In accordance with literature data, analogues containing alicyclic β-amino acids have shown prolonged half-lives ($t_{1/2}$ = > 20 h) in contrast with the parent endomorphins ($t_{1/2}$ = 5-7 min), pointing to the enzyme resistance of the new analogues. These results are summarized in **Table 6**.

Table 6. Half-lives of the endomorphins and their potent analogues in crude rat brain membrane homogenate

no.	peptide	half-life
1	Tyr-Pro-Trp-Phe-NH_2	6.3 ± 0.1 min
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)*β*MePhe-NH_2	>20 h
6	Tyr-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	>20 h
9	Tyr-Pro-Phe-Phe-NH_2	5.7 ± 0.1 min
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)*β*MePhe-NH_2	>20 h
16	Dmt-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	>20 h
17	Tyr-Hyp-Phe-Phe-NH_2	17 ± 0.9 min
18	Tyr-βPro-Phe-Phe-NH_2	34 ± 3 min
19	Tyr-(1*S*,2*R*)ΔAcpc-Phe-Phe-NH_2	> 20 h
21	Tyr-(1*S*,2*R*)ΔAchc-Phe-Phe-NH_2	> 20 h

Data are means of at least three individual experiments ± S.E.M. The protein content of the brain homogenate was 5.0 mg/mL. Half-lives were calculated on the basis of pseudo-first-order kinetics of the disappearance of the peptides.

4.6. *In vitro* BBB permeability of EM-2 analogues by brain capillary endothelial cells

The BBB plays a significant role in the treatment of CNS disorders [30]. It is widely accepted that the mediation of analgesic effects occurs within the CNS, therefore opioid peptides should be able to cross the BBB intact. In order to obtain improved proteolytic stabilities and increased BBB permeabilities, we have synthesized and tritiated three EM-2 analogues: peptide-1 [Dmt-Pro-Phe-Phe-NH_2], peptide-2 [Tyr-(1*S,2R*)Acpc-Phe-Phe-NH_2], peptide-3 [Tyr-(1*S,2R*)Achc-Phe-Phe-NH_2]. These analogues were subjected to asses BBB permeability through rat cerebral endothelial cell layer and the cytotoxic effect of these peptides on endothelial cells were studied. This work was done in collaboration with Dr. István Krizbai and Dr. Imola Wilhelm, Institute of Biophysics, Biological Research Centre, Szeged, Hungary. The series of peptides used in the BBB studies was not specified in the previous chapters. The analogues were chosen based on their improved pharmacological activity evaluated previously in our laboratory [58, 59]. Lipophilicity, is an important factor in the diffusion of the compounds through the BBB. A correlation was observed between the lipophilicity and the structure of the compounds. However, no correlation was observed between lipophilicity and permeabilities. Inhibitory constants (K_i), selectivities, mass spectrometry analysis, half-life and liphophilicites of measured compounds are listed in **Table 7**. The rate of transendothelial transport of the peptides from the luminal (blood) side to the abluminal (brain) side are characterized by the following P_e values: the P_e of EM-2 was $7.84\times10^{-6} \pm 0.73\times10^{-6}$ cm/s (average ± SEM), which was approximately two times higher than the permeability of sodium fluorescein (SF) ($P_e = 3.83\times10^{-6} \pm 0.84\times10^{-6}$ cm/s), which has low BBB permeability and is generally used for the assessment of the integrity of the barrier. The permeability of Evans blue labeled albumin (EBA), which is considered a BBB-impermeable marker, was one order of magnitude lower ($P_e = 7.8\times10^{-7} \pm 2.1\times10^{-7}$ cm/s). All of the analogues showed a significantly increased permeability in comparison with EM-2 (**Figure 6**). In addition to this, cytotoxic effect of these peptides was characterized in brain endothelial cells using a wide range of concentrations

(between 10 nM and 1 mM). None of the peptides affected the viability of the cells significantly, not even in the highest concentrations, suggesting no cytotoxic effect [133].

Table 7. Inhibitory constants (K_i), selectivities, half-life and Log *P* values of EM-2 and its analogues used in the BBB studies

		EM-2	peptide-1	peptide-2	peptide-3
inhibitory constants (nM)	K_i^{μ}	1.3 ± 0.2	0.2 ± 0.08	2.4 ± 0.1	2.4 ± 0.1
	K_i^{δ}	5652 ± 202	93.6 ± 16	> 10000	812 ± 18
selectivity (K_i^{δ}/K_i^{μ})		4348	456	1999	338
MS	$[M]_{cal.}$	571	599	585	599
MS	$[M+H]^+$	572.3	600.3	586.3	600.3
half-life (min)		3.08 ± 0.2	39.6 ± 5.6	> 720	> 720
reference		58	59	58	58
Log *P*		0.457 ± 0.05	1.183 ± 0.03	0.696 ± 0.03	0.925 ± 0.04

[^{3}H]DAMGO (K_d = 0.5 nM) was used as a radioligand for the μ-opioid receptor. [^{3}H]Ile^{5,6}-deltorphin-2 (K_d = 2.0 nM) was used as a radioligand for the δ-opioid receptor. K_i values were calculated according to the Cheng-Prusoff equation: $K_i = EC_{50}/(1+[Ligand]/K_d)$. Data are expressed as means ± SEM, n≥3. Inhibitory constants, half-life, mass spectrometry data was taken from references.

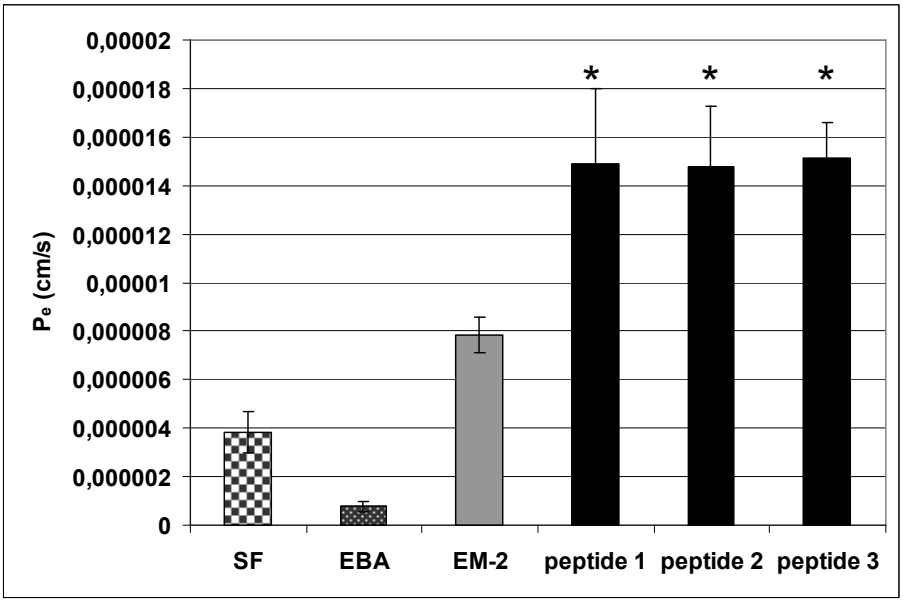


Figure 6. *Permeability coefficient (P_e) of the peptides. The P_e of peptide 1, 2 and 3 was calculated and compared to the permeability of EM-2. As a control the P_e of sodium fluorescein (SF) and Evans blue labeled albumin (EBA) was also calculated. Data are presented as means ± SEM. * = P<0.05 as assessed by ANOVA and Bonferroni's post hoc test (compared to EM-2).*

4.7. *In vivo* characterization of most potent EM-2 analogues

As regards to the *in vivo* antinociceptive effect of endomorphin derivatives, several studies investigated the effects of the ligands after systemic or intracerebroventricular (i.c.v.) administration, while only a few studies are available about the effects of the derivatives at the spinal level. Based on the binding potencies, compounds **10**, **12**, **14**, and **16** were selected to investigate the anti-allodynic effects at spinal level in the chronic joint pain model. *In vivo* studies were performed in collaboration with Dr. Gyöngyi Horváth, Department of Physiology, Faculty of Medicine, University of Szeged, Szeged, Hungary. All of the analogues elicited an

antinociceptive effect in a concentration dependent manner. At low doses (0.1 μg), compounds **12** and **16** produced a significant antinociception, while EM-2 and the other two ligands were ineffective at this dose. Compounds **12** and **16** produced a prolonged antinociception at a 0.3 μg dose while the other ligands were ineffective. In the case of 3 μg, all of the ligands produced anti-allodynia. Regarding the highest dose, 10 μg of compound **16** caused a prolonged paralysis of the animals, therefore, data was not analyzed in the pain test. Morphine, as a positive control, produced long-lasting highly effective antinociception. EM-2, compounds **10** and **14** were as effective as morphine for 10-30 min, while the effect of compounds **12** did not differ significantly from morphine during the whole period (75-120 min). These results were summarized and submitted to *Acta Physiologica Hungarica* journal [134].

4.8. Tritiated neuropeptides

Over the decades tritium labeled bioactive peptides have been used as valuable tools for biological characterization of receptors, and binding sites. The metabolic pathway of tritium labeled compounds is also easily traceable. We are aiming to synthesize biologically active peptides in tritiated form by synthesizing the respective precursor peptide followed by tritiation to acquire suitable tools for use in *in vitro* and *in vivo* biological assays. Several bio-active ligands were tritiated in our laboratory for the past three years to apply them in different biochemical assays. Tritium labeled ligands are very useful in finding the biosynthetic pathways of precursor proteins.

The biosynthetic pathways of other opioid peptides have already been clarified (it happens through a single- or multi-step cleavage from large molecular weight precursor proteins with or without additional post-translational modifications), but the biosynthetic route of endomorphins is still obscure. Recent findings suggested that EM-2 may be produced from a human sulfate transporter protein in consecutive enzymatic reactions [135]. Many biologically active neuropeptides and peptide hormones were shown to be synthesized from inactive glycine-extended precursors, which require a carboxy-terminal

posttranslational amidation for biological activity [135]. This reaction is catalyzed by a consecutive action of peptidylglycine-α-hydroxylating monooxygenase (PHM) and peptidylamidoglycolate lyase (PAL). These two enzymes are part of the peptidylglycine α-amidating monooxygenase (PAM), a bifunctional, integral membrane-bound enzyme machinery [136]. In attempt to elicit the hypothesis that EM-2 may be produced from the sulfate transporter precursor protein by the activation of the PAM enzyme system, we designed and synthesized a novel peptide Gly^5-extended EM-2 and labeled it with tritium. Tritiated Gly^5-EM-2 is being tested in rat brain membrane homogenate. We are still awaiting the results.

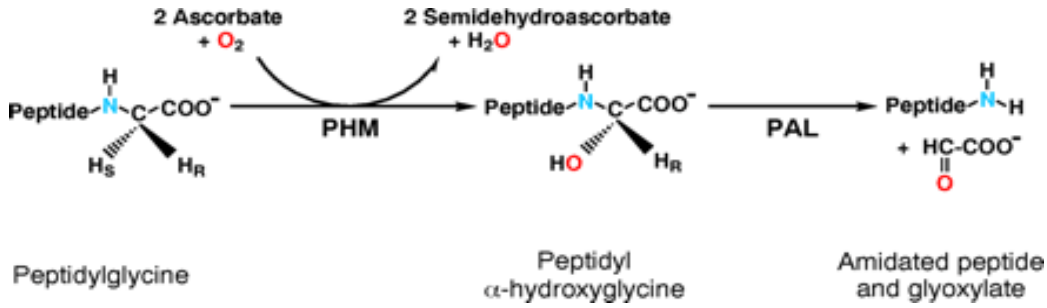


Figure 7. *Reaction catalyzed by bifunctional PAM. The PHM domain catalyzes the oxygen- and ascorbate-dependent hydroxylation of peptidylglycine, forming the α-hydroxyglycine intermediate. The PAL domain cleaves the intermediate and releases amidated peptide and glyoxylate. Colored atoms indicate that molecular oxygen is incorporated into α-hydroxyglycine, and that the product amide nitrogen is derived from the substrate glycine.*

Cumulated data on EM-2 and its analogues and other tritiated neuropeptides was summerized and published recently in *Arkivoc* journal [137]. The following bioactive neuropeptides were tritiated in our laboratory to use in various pharmacological characterizations. Specific activities of tritiated compounds are listed in **Table 8**.

Table 8. Specific radioactivities of tritiated ligands

	Precursor peptides	**Labeled peptides**	**Specific radioactivity (TBq/mmol)**
EM-2 and its analogues	H-Tyr-3,4-ΔPro-Phe-Phe-NH_2	H-Tyr-^{3}**HPro**-Phe-Phe-NH_2	1.87
	H-Dmt-3,4-ΔPro-Phe-Phe-NH_2	H-Dmt-^{3}**HPro**-Phe-Phe-NH_2	2.86
	H-Tyr-(1*S*,2*R*)ΔAcpc-Phe-Phe-NH_2	H-Tyr-**(1*S*,2*R*)^{3}H-Acpc**-Phe-Phe-NH_2	1,41
	H-Tyr-(1*S*,2*R*)ΔAchc-Phe-Phe-NH_2	H-Tyr-**(1*S*,2*R*)^{3}H-Achc**-Phe-Phe-NH_2	2,35
	H-*p*I-Tyr-Pro-Phe-Phe-Gly-OH	H-^{3}**HTyr**-Pro-Phe-Phe-Gly-OH	1.59
Substance P analogues	H-Arg-ΔPro-Lys-ΔPro-Gln-Gln-Phe -Phe-Gly-Leu-Nle-NH_2	H-Arg-^{3}**HPro**-Lys-^{3}**HPro**-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2	0,84
	H-Arg-ΔPro-Lys-ΔPro-Gln-Gln-Phe-OH	H-Arg-^{3}**HPro**-Lys-^{3}**HPr**o-Gln-Gln-Phe-OH	1,09
Neuromedin N	H-Lys-Ile-ΔPro-Tyr-Ile-Leu-OH	H-Lys-Ile-^{3}**HPro**-Tyr-Ile-Leu-OH	0.6
Angiotensin IV analogues	H-(R)-β^{2}hVal-Tyr-Ile-His-ΔPro-β^{3}hPhe-OH	H-(R)-β^{2}hVal-Tyr-Ile-His-^{3}**HPro**-β^{3}hPhe-OH (^{3}H-AL-11)	1,29
	H-(R)-β^{2}hVal-Tyr-Ile-Aia-Pra-Phe-OH (IVDE76)	H-(R)-β^{2}hVal-Tyr-Ile-Aia-^{3}**HNva**-Phe-OH (IVDE77)	0.9
Nociceptin	H-[4-I]Phe-Gly-Gly-pFPhe-Thr-Gly-Aib-Arg-Lys-Ser-Ala-Arg-Lys-Arg-Lys-Asn-Gln-NH_2	H-**[4-^{3}H]Phe**-Gly-Gly-pFPhe-Thr-Gly-Aib-Arg-Lys-Ser-Ala-Arg-Lys-Arg-Lys-Asn-Gln-NH_2 (^{3}H-UFP112)	0.73

5. Discussion

In the structure of endomorphins three aromatic amino acid residues, which play a crucial role in the recognition of the opioid receptors, are connected by a Pro^2 residue. This residue is considered a spacer, stabilizing the bioactive conformation of the endomorphins [19, 58]. The extensively investigated *cis-trans* isomerization around the Tyr^1-Pro^2 peptide backbone permits the endomorphins to adopt many accessible conformations, making the determination of bioactive conformation(s) and the mode of ligand binding difficult [48]. The backbone and side-chain conformations of the μ-opioid receptor ligands contribute concomitantly to the orientation of the pharmacophore groups. To reveal the precise structural orientations and interactions between the μ-opioid receptor and endomorphins, numerous analogues needed to be synthesized. In the present study, unnatural amino acids were introduced into different positions of endomorphins in order to obtain proteolytically stable compounds for the study of the bioactive structure(s), favorable for opioid binding. To obtain the desired analogues, Dmt^1, constrained alicyclic β-amino acids in the place of Pro^2, side chain constrained $\beta MePhe^4$ and $pFPhe^4$ in the position of Phe^4 were inserted. Dmt substitution of the Tyr^1 residue yielded potent agonist analogues with high affinities and various selectivities [53, 59, 138 and 139]. The observed variabilities in biological properties may be due to interactions between Dmt^1 and the remaining structural elements: the Tyr^1-Pro^2 and aromatic-aromatic interactions (Tyr^1/Dmt^1-Trp^3, -Phe^3, -Phe^4). They were found to be involved in stabilizing the local conformations of endomorphins [82]. Furthermore, inserted methyl groups cause decreased acidity of the phenolic hydroxyl group, accordingly optimizing the hydrophobic and steric interactions of the analogues with the opioid receptors [58]. Consequently, the substituted analogues retain their affinities for the μ-opioid receptors, though they suffer a remarkable loss in selectivity.

Incorporation of Dmt, racemic *cis*- alicyclic β-amino acids, Hyp, βPro, side chain restricted βMePhe and pFPhe yielded a series of peptide analogues with altered proteolytic stabilities and pharmacological activities. Compounds with (1*S*,2*R*)-β-amino acid configuration were found to be more potent than those of the (1*R*,2*S*) configuration.

Substitution with para-halogenated amino acids [among all halogenated (F, Cl, Br and I) Phe^4 residues] $pFPhe^4$ residue containing EM-1 and -2 derivatives showed an increased affinities for μ-opioid receptor (K_i = 1.2 nM and 3.4 nM for $pFPhe^4$-EM-1 and $pFPhe^4$-EM-2 respectively) in receptor binding assays compared to other halogenated analogues. Endomorphins with $pClPhe^4$ displayed K_i values for μ-opioid receptor 3.3 nM and 7.4 nM for EM-1 and -2 respectively. Analogues with $pBrPhe^4$ showed K_i values with 11 nM and 13 nM for EM-1 and -2 respectively, for the μ-opioid receptor. $pIPhe^4$ containing analogues exhibited K_i values with 11nM and 39 nM for EM-1 and EM-2 respectively, for the μ-opioid receptor. All of the halogenated analogues resulted in decreased selectivities [67]. The present findings confirmed the selectivity of halogenated amino acids in the place of Phe^4 to design biologically active endomorphins. Incorporation of (2*S*,3*S*)βMePhe resulted in analogues with increased affinity and potency as compared with the parent ligands, while (2*R*,3*R*)βMePhe substitution decreased the affinity and selectivity toward the μ-opioid receptor. These results confirm that the modulation of side chain rotations is a very important feature in determining the biological activity of a peptide. These results further confirmed the importance of chirality in binding to the receptor [58, 68]. Insertion of (*Z*)-*α*, β-didehydrophenylalanine ([Δ^zPhe]-EM-2 in the position of Phe^3, Phe^4 in EM-2 resulted in a different binding affinity profile. The [$\Delta^z Phe^4$]-EM-2 exhibited higher μ-receptor affinity (K_i=8.4 nM) and selectivity ($K_i^{\delta/\mu}$= >1200) than that of the [$\Delta^z Phe^3$]-EM-2 (K_i=200 nM) [140]. Similar results were observed for substitutions with βMePhe in the place of Phe^3, Phe^4 in EM-2 which further confirmed the significance of Phe^3 in endomorphins [141]. Morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2), another μ-opioid receptor-selective agonist with identical N-terminal message sequence to endomorphins, provided important clues regarding its binding activity and specificity [87]. The insertion of diastereomric βPro^2 in morphiceptin yielded analogues with different biological activity profile. In contrast with the present results, (*R*)-βPro^2 had higher μ-opioid receptor affinity (K_i=58 nM) than the other isomer (*S*)-βPro^2 (K_i=293 nM), both analogues were inactive at the δ-opioid receptor [87]. These results confirm that each class of opioid peptides shows distinct chiral requirements for the spacers between the biologically important Tyr and Phe residues [47]. Introduction of a polar -OH group on the Pro^2 side chain and the extension of the backbone by a -CH_2-

group, while preserving the tertiary amide moiety of Pro has disadvantageous effects on μ-opioid receptor activity of EM-2 in general, regardless of different experimental conditions [142]. However, the observed effect of βPro^2 substitution may not be applicable generally for all μ-opioid receptor ligands, since in a previous study βPro-EM-1 exhibited μ-opioid receptor affinity, efficacy and agonist behavior similar to those of DAMGO and EM-1 [130]. This indicates that the incorporation of a -CH_2- group between the pyrrolidine ring and the carboxyl group of the second residue of the endomorphin sequence may affect receptor-ligand interactions differently when the following amino acid residue is Trp or Phe. Furthermore, diastereomeric βPro^2 substitution in morphiceptin yielded analogues with different biological activity profile, (*R*)-βPro^2-morphiceptin had higher μ-opioid receptor affinity than (*S*)-βPro^2-morphiceptin [87]. This suggests that in certain cases μ-opioid receptor ligands may have distinct chiral requirements for the spacers between the biologically important Tyr and Phe residues. In endomorphin analogues containing unsaturated alicyclic β-amino acids the 1*S*,2*R* side chain configuration was shown to furnish μ-opioid receptor activity exclusively.

The ligand stimulated [^{35}S]GTPγS binding assay is a common pharmacological probe that is widely used to determine functional properties of receptor ligands. As this assay measures the level of G-protein activation following agonist occupation of the binding site, its advantage is that it follows the direct functional consequences of receptor occupancy, independently of the downstream-effector systems [143, 144]. In a pool of analogues, most of the endomorphin derivatives exhibited higher efficacies than those of the parent compounds. Based on the [^{35}S]GTPγS binding assay results, all examined endomorphin analogues retained partial agonist properties, similar to those of the endomorphins. Insertion of alicyclic β-amino acid itself did not alter the agonist/antagonist properties in both endomorphins [58]. The co-application of Dmt, alicyclic β-amino acid (*cis*-(1*S*,2*R*)Achc) and pFPhe resulted in a highly potent analogue. The observed efficacy for compound **16** (E_{max}=176%) was comparable with the universal μ-receptor agonist DAMGO (E_{max}=178%). The results of the [^{35}S]GTPγS binding experiments in the presence of the general antagonist naloxone showed that the ligands bind to opioid receptors, activate G-proteins and presumably downstream signaling. It is

interesting to note that compounds **4**, **12** and **16** stimulated G-protein binding even in the presence of 10^{-5} M naloxone (data not shown) therefore, we hypothesized that these ligands may also bind to other G-protein coupled receptors or to non-opioid binding sites.

Highly potent analogues were selected based on receptor binding and [^{35}S]GTPγS binding assays to perform metabolic stability studies of the newly synthesized analogues against proteolytic degrading enzymes. Analogues containing Hyp^2 and βPro^2 had increased 3- and 6 fold higher stabilities, respectively in comparison with the parent compound EM-2. In consistence with earlier results [58, 79], analogues with alicyclic β-amino acids have shown prolonged half-lives (>20 h) compared to parent endomorphins ($t_{1/2}$=5-7 min). These results confirmed that Pro^2 targeting modifications result in proteolytically stable peptides.

^{1}H-NMR studies on Dmt^1 substituted endomorphins showed higher *cis* conformation (*cis*/*trans* ratio 2:1) [59], this conformational differing from that calculated for parent compounds (higher *trans* ratio) were based on molecular modeling studies [81]. When the results are taken into account, a reduction of binding potency can be expected, but both analogues displayed comparable binding to endomorphins in rat brain membrane preparations [59]. Thereby, the interpretation of ^{1}H-NMR and molecular modeling data demands caution. To control the *cis/trans* isomerization around the Tyr^1-Pro^2 peptide backbone, alicyclic β-amino acids were inserted in the place of Pro^2. The incorporation of racemic alicyclic β-amino acids resulted in analogues with altered biological activities depending on chirality of amino acid [58, 79]. Previous studies on substitution of Pro^2 by (1*S,*2*R*)Acpc^2 in endomorphins were shown to adopt bent structure more readily than (1*R,*2*S*)-Acpc-containing analogues [58]. In consistence with previous findings, ^{1}H-NMR and molecular modeling studies on highly potent analogues have shown a higher tendency to form a bent structure, suggesting turn structure for μ-opioid receptor binding. In addition to this, various classic and non-canonical turn structures were identified as possible conformations of the analogues studied by the analysis of specific intramolecular H-bonds. The identified conformations provide advantageous orientation of the pharmacophore groups resulting in high-affinity binding to the μ-opioid

receptor [81]. Several unique structural elements were identified as the consequence of the elongation of the peptide backbone by incorporation of a β-amino acid. As biological data suggests, this elongation does not significantly affect the folding of these peptides or the orientation of the pharmacophore elements, confirming the role of Pro^2 as a stereochemical spacer [79]. This is in accordance with previous findings on morphiceptin analogues with constrained topologies, where several unique structural elements were found as consequences of elongation of the peptide chain due to application of alicyclic β-amino acids [70]. Most of the special, non-canonical secondary structural elements were found to be stabilized by intramolecular H-bonds. In addition, aromatic–aromatic and proline–aromatic interactions may also play a significant role in the stabilization of such structures [81, 145].

The combined analysis of the competitive receptor binding assay, [^{35}S]GTPγS binding assay and ^{1}H-NMR studies and molecular dynamics simulations suggest that the receptor-ligand interactions are regulated by diverse structural aspects after the introduction of unnatural amino acids. Variability of the binding affinities and selectivities of the ligands could be explained, with the assumption that upon ligand binding to the receptor it might generate different affinity states in opioid receptors by either the *cis* or *trans* isomers of the opioid ligands. 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 [88, 90 and 146]. In addition to this, it can't be excluded that ligands may bind to different subtypes of the μ-receptor, i.e. hypothetical μ_1- and μ_2- receptors [147] which might be distinct proteins, or different binding proteins of the same protein. Although such distinction still waits in the case of μ-receptor, the results of the present study describing the synthesis and binding characteristics of endomorphin analogues contribute in understanding structural and topographical requirements of peptide ligand binding.

Several endomorphin and neuropeptide analogues were tritiated in our laboratory to use as a tool in different biochemical assays such as *in vitro* radioligand receptor

binding assays, radioimmunoassays. The advantage of tritium labeled radioactive peptides is that the structure of the radioactive material is the same as the parent peptide, so the biological activity is identical. Tritiated ligands are used in finding biosynthetic pathways of precursor proteins; in homologue or heterologue displacement studies to characterize the new derivatives.

Although endomorphins are very potent and highly selective μ-opioid receptor agonists, their systemic administration for the clinical treatment of pain is impeded by their rapid degradation and limited delivery to the CNS. Promising structural modifications of both EM-1 and EM-2 have already been described [52, 53, 58, 59 and 62]. In these studies an improved antinociception was observed following peripheral administration of the peptide, which may be attributed to improved brain delivery, increased resistance against degradation or both. Evaluated EM-2 analogues [Dmt-Pro-Phe-Phe-NH_2, Tyr-(1*S,2R*)Acpc-Phe-Phe-NH_2, Tyr-(1*S,2R*)Achc-Phe-Phe-NH_2] showed relatively low, but improved BBB permeability properties compared to the parent compound EM-2. None of the peptides had a cytotoxic effect on the viability of brain endothelial cells. Accumulating the results of BBB permeabilities, antinociceptive effect and their enhanced enzymatic resistance, these peptides will have better analgesic properties *in vivo* and can lead to the development of successful opioid drugs.

A series of analogues, containing non-cyclic amino acids (Ala, D-Ala, β-Ala, N-methylalanine: NMeAla, D-NMeAla or N-methylglycine) instead of Pro in position 2 were synthesized to assess the analgesic properties of endomorphins [148]. In the *in vivo* test applied in mice (HP test, i.c.v. administration) D-Ala^2- EM-2 and D-$NMeAla^2$- EM-2 showed much higher analgesic potency than EM-2 which confirmed the usefulness of structural modifications in obtaining new leads for pain-relief therapeutics. EM-2 cyclic asparagine analogues (H-Tyr-**c**Asp[CHPh]-Phe-Phe-NH_2 and H-Tyr-**c**Asp [$CHMe_2$]-Phe-Phe-NH_2) were investigated in acid-induced writhing test in mice after systemic (i.p.) administration [149]. H-Tyr-**c**Asp (CHPh)-Phe-Phe-NH_2 had a higher potency compared to the parent drug, while H-Tyr-**c**Asp ($CHMe_2$)-Phe-Phe-NH_2 had a lower potency. Endomorphin analogues with six-membered heterocyclic rings (Tyr-[*R*]-Nip-Phe-Phe-

NH_2; Dmt-Nip-Phe-Phe-NH_2; [Nip: piperidine-3-carboxylic acid]) were investigated in HP in mice, after i.c.v. administration [150]. These ligands produced a profound, long-lasting supraspinal analgesia, being much more potent than EM-2. An earlier study showed that dimethyl-analogue of EM-2 (Dmt-EM-2) produced antinociception after i.t. injection in formalin test (rat) [151]. Among the tested analogues compounds **10** and **14** had similar effects than EM-2, and this is in agreement with their K_i values for the μ-opioid receptor too. Compounds **12** and **16** showed high affinity to the μ-opioid receptor and these ligands also had a long half-life in crude rat brain membrane homogenate. Therefore, the activation of the μ-opioid receptor and their high metabolic stability could have led to prolonged antinociception. The results demonstrate that complex modification of endomorphins by introduction of Dmt, alicyclic β-amino acids, βMePhe, and pFPhe into different position in EM-2 might be a promising strategy to enhance bioavailability of the peptides and may serve a role in the development of novel endomorphin analogues with increased therapeutic potential. Further studies are required to reveal the possible side effects of these ligands.

6. Summary of findings

- A small pool of endomorphin analogues were synthesized by systematic incorporation of unnatural amino acids, such as Dmt[1], Achc[2], ΔAcpc[2]/ΔAchc[2], Hyp[2], βPro[2], βMePhe[4] and pFPhe[4]. Such modifications yielded the analogues with increased proteolytic stability while retaining or enhancing the biological activity.

- Radioligand receptor-binding studies of newly synthesized analogues revealed that insertion of these amino acids resulted in moderate to highly potent compounds depending on the chiralities of the incorporated amino acids. The multiple modifications yielded the analogues with increased μ-receptor affinities, but with decreased selectivities.

- The ligand-stimulated [^{35}S]GTPγS binding assay results revealed that some of the analogues showed higher efficacies than the parent endomorphins. Most of the compounds retained agonist or partial agonist properties.

- Combined application of Dmt[1], *cis*-(1*S*,2*R*)Achc[2] and pFPhe[4] resulted in the most potent analogue with a 1 order of magnitude higher receptor affinity compared to the parent compound EM-2. Furthermore, it displayed the highest efficacy in the ligand-stimulated [^{35}S]GTPγS binding assay, acting as a full agonist.

- Findings of this study revealed that unsaturation of alicyclic β-amino acids had no effect on binding affinities of EM-2 in comparison with saturated alicyclic β-amino acids.

- The compound bearing *cis*-(1*S*,2*R*)ΔAcpc[2] displayed an equal μ-opioid receptor affinity with high selectivity compared to the parent compound EM-2.

- Dmt[1], (1*S*,2*R*)Acpc[2], and (1*S*,2*R*)Achc[2] containing EM-2 analogues have displayed higher blood-brain barrier permeabilities in comparison with the parent ligand with no viability of endothelial cells. None of the tested ligands had a cytotoxic effect on viability of rat brain endothelial cells.

- All of the tested ligands elicited an antinociceptive effect in a concentration dependent manner. Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2 elicited antinociception comparable with morphine.

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9. Összefoglaló

Az opioidok kutatásának fő célja hatékony fájdalomcsillapítók kifejlesztése, amelyek az opiátok káros mellékhatásait (fizikai függőség, tolerancia és légzés depresszió) kiküszöbölhetik és hatékonyan helyettesíthetik a morfint. Marha agyból majd később humán agyból izolált két endogén peptid, endomorfin-1 és -2 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2; EM-2, H-Tyr-Pro-Phe-Phe-NH_2) nagy affinitással és szelektivitással kötődik a μ-opioid receptorokhoz. Mivel az endomorfinok nagy affinitással kötődnek a μ-opioid receptorokhoz, és ezen receptorok felelősek a fájdalomérzékeléséért és modulációjáért, ezen tetrapeptidek fontos modellek lettek a fájdalomcsillapítók kifejlesztésének kutatásában. Az endomorfinok használatát számos tényező befolyásolja, pl. rövid hatástartam, hatástalan ok *per os* adagolás esetén, a kotlátozoth vér-agy gáton való átjutások és a proteolitikus enzimekkel szemben metabolikus stabilitásuk gyenge. A korlátozó tényzők leküzdése érdekébeni több nem természetes aminosavval (Dmt[1], *cisz*-(1*S*,2*R*)Achc[2]/*cisz*-(1*R*,2*S*)Achc[2], *cisz*-Δ(1S,2R)Acpc[2], *cisz*-Δ(1S,2R)Achc[2], Hyp[2], βPro[2], (2*R*,3*R*)βMePhe[4]/(2*S*,3*S*)βMePhe[4]) szubsztituált endomorfin analógot szintetizáltunk. Az így szubsztituált endomorfin analógok a proteolitikus stabilitás növekedéséhez vezettek, miközben megtartották vagy növelték azok biológiai aktivítását.

A peptid szintézist Boc védőcsoporttal ellátott aminosavakkal 4-Metil-benzhidrilamin gyantán, szilárd fázisú peptidszintézis módszerével valósítottuk meg. Az új endomorfin analógok hatékonyságának és szelektivitásának elemzése patkány agyi membránpreparátumon radioligand-kötési teszttel történt. A μ-receptor affinitás méréshez [^{3}H]DAMGO-t, míg a δ-receptor affinitás méréshez [^{3}H]Ile^{5,6}-deltorphin II-t használtunk. Az új vegyületek koncentráció függő módon versengtek a radiojelzett μ- és δ-receptor ligandumokkal a receptor kötési helyekért. [^{35}S]GTPγS mérésekkel határoztuk meg a ligandumok funkcionális tulajdonságait.

A receptor kötési vizsgálatok analízise kimutatta hogy mindkét endomorfin esetében a (1*S*,2*R*)Achc[2] és a (2*S*,3*S*)βMePhe[4] együttes használata az eredeti peptidekhez képest hasonlóan potens analógokhoz vezetett. A kapott eredmények alátámasztották,

hogy a Dmt[1] szubsztitució fokozza a μ-opioid receptor affinitást, miközben csökkenti a ligandumok szelektivitását. A (2*R*,3*R*)βMePhe[4]-al szubsztituált analóg kisebb affinitást mutatott, mint a másik izomer a (2*S*,3*S*)βMePhe[4] tartalmú peptid. Az Achc[2] és a pFPhe[4] együttes szubsztituciója olyan analógokat eredményezett, melyek bioaktivitása az aliciklikus β-aminosavak kiralitásától függött. Az összes analóg közül a Dmt[1], (1*S*,2*R*)Achc[2], pFPhe[4]-endomorfin-2 rendelkezett a legnagyobb μ-opioid receptor affinitással (K_i = 0.13 nM).

Hyp[2] szubsztitució csökkentette μ-opioid receptor affinitást (K_i = 44 nM), A βPro[2] tartalmú analóg alacsonyabb affinitáshoz vezetett (K_i = 27 nM) az eredeti endomorfin-2 – höz képest (K_i = 1.35 nM). A peptidgerinc CH_2-csoporttal való meghosszabítása (βPro), a Pro tercier amid molekularész megőrzése mellett, nem előnyös az endomorfin-2 μ-receptor aktivitására. A βPro[2] szubsztitució esetében észlelt hatás nem biztos hogy általánosan alkalmazható minden μ-receptor ligandumra, mivel egy korábbi vizsgálat során a βPro-endomorfin-1 a DAMGO-hoz és az endomorfin-1-hez hasonló μ-receptor affinitást, "efficacy" profilt és agonista karaktert mutatott. A -CH_2-csoport beillesztése a pirrolidin gyűrű és a kaboxil csoport közé (βPro) az endomorfinban, másképpen befolyásolja a receptor-ligandum kölcsönhatást, ha a következő aminosav Trp vagy Phe. A *cisz*-(1*S*,2*R*)ΔAcpc[2]/*cisz*-(1*S*,2*R*)ΔAchc[2] tartalmú analógok az eredeti endomorfin-2-höz hasonló affinitást mutattak. A telítetlen aliciklikus β-aminosav tartalmú endomorfin analógok kötési affinitása összehasonlítható a telített β-aminosav tartalmú endomorfin analógokéval. A *cisz*-(1*S*,2*R*)ΔAcpc[2]-endomorfin-2 azonos affinitást (K_i = 1.3 nM) és nagyobb μ-opioid receptor szelektivítást mutatott a eredeti peptidhez képest.

A heterológ leszorítási kisérletek eredményei alapján választottuk ki a legpotensebb analógokat a [^{35}S]GTPγS mérésekhez. A potencia (EC_{50}) és a hatásfok (efficacy, E_{max}) értékekeket a tiszta agonista DAMGO értékeihez hasonlítottuk. A vegyületek dózis függő módon stimulálták a funkcionális [^{35}S]GTPγS kötést. Az endomorfin-1 és az endomorfin-2 a DAMGO-hoz hasonló EC_{50} értéket, de alacsonyabb hatásfokot mutat, mely alátámasztja hogy az endomorfinok parciális agonisták. A *cisz-(*1*S*,2*R*)ΔAcpc[2], *cisz*-(1*S*,2*R*)ΔAchc[2], (1*S*,2*R*)Achc[2], és (2*S*,3*S*)βMePhe[4] tartalmú

analógok nagyobb hatásfokot mutattak, tiszta agonistaként viselkednek. Hyp^2 szubsztitució az endomorfin-2-ben alacsonyabb hatásfokot eredményezett, mely mérsékelt agonista hatást sugall. A βPro^2 tartalmú analóg szolgáltatta a legalacsonyabb hatásfokot, gyenge agonistaként viselkedik. A naloxon jelenlétében elvégzett [^{35}S]GTPγS mérések eredménye kimutatta hogy a ligandumok opioid receptorokon keresztül aktiválják a G-proteineket.

A biológiai aktivitási eredmények alapján a hatékony analógokon stabilitási vizsgálatokat végeztünk patkányagyi membrán preparatumon. Az (1*S*,2*R*)$Achc^2$ tartalmú analógok felezési ideje (>20 óra) jelentősen emelkedett az eredeti endomorfinok felezési idejéhez ($t_{1/2}$ = 5-7 perc) képest, mely az új analógok enzim rezisztenciáját bizonyítja. Az endomorfin-2 esetében a Hyp^2 szubsztitúció 3x-os, a βPro^2 beépítése 6x-os növekedést eredményezett a proteolitikus stabilitásban az eredeti vegyülethez képest. Ezen eredmények alátámasztják, hogy az aliciklikus β-aminosavak beépítése proteolitikusan stabil analógokhoz vezet.

Laboratóriumunkban számos tríciált endomorfin-, és neuropeptid analógot állítottunk elő, különböző biokémiai vizsgálatokhoz. A tríciummal jelzett radioaktív peptidek előnye, hogy a radioaktív anyag szerkezete megegyezik az eredeti peptidével, így a biológiai aktívitásuk is hasonló. A tríciált ligandumokat felhasználtuk prekurzor proteinek bioszintetikus útvonalának keresésére és homológ vagy heterológ leszorítási kísérletekben az új analógok biológiai jellemzésére.

A leghatékonyabb endomorfin-2 analógokat *in vivo* krónikus izületi fájdalom modelben spinális szinten vizsgáltuk az anti-allodinikus hatások felderítésére. Az allodynia egy olyan állapot, amelyben egy rendesen nem fájdalmas inger, fájdalmat vált ki. Morfint használtunk kontrollnak. Az összes analóg koncentráció függő módon váltott ki antinociceptív hatást. Ezen eredmények azt sugallják, hogy a Dmt, az aliciklikus β-aminosavak, a βMePhe, és a pFPhe aminosavak beépítése az endomorfin-2-ben egy ígéretes stratégia lehet a peptidek biohasznosíthatóságának növelésére, és szerepet játszhat új endomorfin analógok kifejlesztésében, melyek fokozott terápiás lehetőséggel

bírnak. Mindamellett további vizsgálatok szükségesek ezen ligandumok lehetséges mellékhatásainak felderítésére.

Korábbi eredmények alapján ismert hogy az endomorfinok hatékony, nagy szelektívivású μ-opioid receptor agonisták. A gyenge metabolikus stabilitás és a korlátozott vér-agy gáton való átjutás, mind eimitálja a fájdalom klinikai kezelését endomorfinok szisztémás adásával. Az endomorfin-1-ben és endomorfin-2-ben végrehajtott számos ígéretes szerkezeti módosítás megnövekedett antinocicepcióhoz vezetett, melyet a peptid perifériás beadása után észleltek, ez a hatás a jobb vér-agy gát permeablitásnak vagy a megnövekedett degradáció elleni rezisztenciának vagy mindkettőnek tulajdonítható. A vizsgált endomorfin-2 analógok [Dmt-Pro-Phe-Phe-NH_2, Tyr-(1*S,2R*)Acpc-Phe-Phe-NH_2, Tyr-(1*S,2R*)Achc-Phe-Phe-NH_2] viszonylag alacsony, de jobb vér-agy-gát permeabilitást mutattak az eredeti peptidhez képest. Az agyi endotél sejtek életképességére egyik vizsgált peptid sem hatott citotoxikusan. A jobb vér-agy gát permeablitás, az antinociceptiv hatás és a megnövekedett enzimatikus rezisztencia mind azt bizonyítja hogy ezen peptidek prekurzorként szolgálhatnak opioid gyógyszerek fejlesztésében.

10. Summary

A major goal in opioid research is the development of novel analgesics having fewer side effects (physical dependence, tolerance and respiratory depression), that can substitute morphine. Two endogenous (Endomorphins: EM-1, H-Tyr-Pro-Trp-Phe-NH_2; EM-2, H-Tyr-Pro-Phe-Phe-NH_2) peptides were isolated from bovine brain and subsequently from the human central nervous system with a high affinity and selectivity for μ-opioid receptors. Since endomorphins have high affinity for μ-opioid receptors and these receptors mediate many of biological functions that include pain perception and modulation, these tetrapeptides became important model peptides in the research towards analgesics. Exogenous application of endomorphins suffers from serious limitations, including short duration of action, lack of activity after oral administration, relative inability to cross the blood-brain barrier into the central nervous system and poor metabolic stability. In order to overcome the afore mentioned objectives, we synthesized a pool of endomorphin analogues by incorporating unnatural amino acids, such as Dmt^1, *cis*-(1*S*,2*R*)$Achc^2$/*cis*-(1*R*,2*S*)$Achc^2$, *cis*-Δ(1S,2R)$Acpc^2$, *cis*-Δ(1S,2R)$Achc^2$, Hyp^2, βPro^2, (2*R*,3*R*)$\beta MePhe^4$/(2*S*,3*S*)$\beta MePhe^4$, $pFPhe^4$. Such modifications yielded analogues with an increased proteolytic stability while retaining or enhancing the biological activity.

Peptide synthesis was carried out by manual solid phase peptide synthesis method on 4-methylbenzhydrylamine resin using Boc-protected amino acids. The potency and selectivity of the newly synthesized endomorphin analogues were evaluated by means of radioligand binding assays in rat brain membrane homogenate. [^{3}H]DAMGO was used to measure the μ-receptor affinity, [^{3}H]$Ile^{5,6}$-deltorphin-2 was applied to evaluate the δ-receptor affinity. All the new compounds competed with the radiolabeled μ- and δ-receptor ligands in a concentration dependent manner for the receptor binding sites. The functional properties were evaluated using [^{35}S]GTPγS binding assays.

Analysis of the receptor binding data revealed that co-application of (1*S*,2*R*)$Achc^2$ and (2*S*,3*S*)$\beta MePhe^4$ in both endomorphins resulted in comparably potent analogues relative to the native peptides. The obtained results confirmed that the use of Dmt^1

augments the affinity for μ-opioid receptors but, at the same time, decreases selectivity of the ligands. Analogues containing corresponding (2*R*,3*R*)βMePhe^4 residue exhibited lower affinities than those with the other isomer (2*S*,3*S*)βMePhe^4. Co-substitution of Achc^2 with pFPhe^4 yielded analogues with diverse bioactivities depending on the chirality of the alicyclic β-amino acids. Among all, the analogue containing Dmt^1, (1*S*,2*R*)Achc^2 and pFPhe^4 displayed the highest μ-opioid receptor affinity (K_i = 0.13 nM) with high selectivity. Incorporation of Hyp^2 resulted in a decreased affinity to μ-opioid receptors (K_i = 44 nM), in contrast to affinity values measured with the radiolabeled opioid antagonist naloxone in crude rat brain membrane. Insertion of βPro^2 resulted in lower affinity (K_i = 27 nM) compared to that of parent EM-2 (K_i = 1.35 nM). Insertion of a polar -OH group on the Pro^2 side chain and the extension of the backbone by a -CH_2- group, while preserving the tertiary amide moiety of Pro has disadvantageous effects on μ-receptor activity of EM-2. However, the observed effect of βPro^2 substitution may not be applicable generally for all μ-receptor ligands, since in a previous study βPro-EM-1 exhibited μ-receptor affinity, efficacy and agonist behavior similar to those of DAMGO and EM-1. This indicates that the incorporation of a -CH_2- group between the pyrrolidine ring and the carboxyl group of the second residue of the endomorphin sequence may affect receptor-ligand interactions differently when the following amino acid residue is Trp or Phe. Furthermore, diastereomeric βPro^2 substitution in morphiceptin yielded analogues with different biological activity profile, (*R*)βPro^2-morphiceptin had higher μ-receptor affinity than (*S*)βPro^2-morphiceptin. Analogues bearing *cis*-(1*S*,2*R*)ΔAcpc^2/*cis*-(1*S*,2*R*)ΔAchc^2 displayed comparable affinities relative to the parent EM-2. The observed propensity of binding affinities for unsaturated alicyclic β-amino acid containing analogues is comparable with those of saturated alicyclic β-amino acid containing endomorphin analogues. Ligand with *cis*-(1*S*,2*R*)ΔAcpc^2 exhibited equal affinity (K_i = 1.3 nM) and higher selectivity for μ-opioid receptor relative to parent EM-2.

On the basis of the heterologous displacement binding results, the most potent analogues were selected for [^{35}S]GTPγS binding assays. Potency (EC_{50}) and efficacy (E_{max}) values were compared with those of the μ-receptor full agonist DAMGO. Dose-dependent increases were observed for selected compounds in [^{35}S]GTPγS binding. Both

parent ligands exhibited comparable potencies, but lower efficacies were observed as compared to DAMGO, confirming that endomorphins are partial agonists. Analogues containing *cis*-(1*S*,2*R*)ΔAcpc^2, *cis*-(1*S*,2*R*)ΔAchc^2, (1*S*,2*R*)Achc^2, and (2*S*,3*S*)βMePhe^4 displayed higher efficacies, acting as full agonists. Substitution of Hyp^2 in EM-2 resulted in lower efficacy, suggesting it as a moderate agonist. Incorporation of βPro^2 yielded the analogue with lowest efficacy, behaving as a weak agonist. The analysis of [^{35}S]GTPγS binding assay results, performed in the presence of naloxone, revealed that the ligands predominantly activate G-proteins through opioid receptors.

Based on biological data, highly potent analogues were selected and their resistance against proteolytic enzymatic degradation was evaluated. The analogues containing (1*S*,2*R*)Achc^2 were shown to have prolonged half-lives (>20 h) in contrast with the parent endomorphins ($t_{1/2}$ = 5-7 min), pointing to the enzyme resistance of the new analogues. Incorporation of Hyp^2 and βPro^2 increased the proteolytic stability 3- and 6 fold higher, respectively, compared to the parent compound EM-2. These results confirmed that insertion of alicyclic β-amino acids results in proteolytically stable analogues.

Several endomorphin and neuropeptide analogues were tritiated in our laboratory to use as a tool in different biochemical assays such as *in vitro* radioligand receptor binding assays, radioimmunoassays. The advantage of tritium labeled radioactive peptides is that the structure of the radioactive material is the same as the parent peptide, so the biological activity is identical. Tritiated ligands are used in finding biosynthetic pathways of precursor proteins; in homologue or heterologue displacement studies to characterize the new derivatives.

Highly potent EM-2 analogues were selected to investigate anti-allodynic effects at spinal level in *in vivo* chronic joint pain model. Allodynia is a pain due to a stimulus which does not normally provoke pain. Morphine was used as a positive control. All of the analogues elicited an antinociceptive effect in a concentration dependent manner. These results suggest that insertion of Dmt, alicyclic β-amino acids, βMePhe, and pFPhe

into different positions in the EM-2 might be a promising strategy to enhance bioavailability of peptides and may serve a role in the development of novel endomorphin analogues with increased therapeutic potential. However, further studies are required to reveal the possible side-effects of these ligands.

As previously described endomorphins are potent and highly selective μ-opioid receptor agonists. The clinical treatment of pain with systemic administration of endomorphins is impeded by their poor metabolic stability and limited ability to cross the BBB. Several promising structural modifications of both endomoprhin-1 and -2 resulted in improved antinociception that was observed following peripheral administration of the peptide, which may be attributed to improved brain delivery, increased resistance against degradation or both. Here, the evaluated EM-2 analogues [Dmt-Pro-Phe-Phe-NH_2, Tyr-(1*S,2R*)Acpc-Phe-Phe-NH_2, Tyr-(1*S,2R*)Achc-Phe-Phe-NH_2] were found to have relatively low, but improved blood-brain-barrier permeability properties compared to the native endmorphin-2. None of the peptides had shown cytotoxic effects on the viability of the brain endothelial cells. The combined results of blood-brain barrier permeabilities, antinociceptive effect and their enhanced enzymatic resistance demonstrate that these peptides can serve as precursors for the development of successful opioid drugs.

11. List of publications

11.1. List of thesis related publications

2011:

Mallareddy, J. R.; Borics, A.; Keresztes, A.; Kövér, K. E.; Tourwé, D.; Tóth.G. *Design, Synthesis, Pharmacological Evaluation, and Structure-Activity Study of Novel Endomorphin Analogues with Multiple Structural Modifications.*
Journal of Medicinal Chemistry, 54: 1462-1472.
Impact Factor: **5.207**

2012:

Mallareddy, J. R.; Tóth, G.; Fazakas, C.; Molnár, J.; Nagyőszi, P.; Lipkowski, A. W.; Krizbai, I.A.; Wilhelm, I. *Transport Characteristics of Endomorphin-2 Analogues in Brain Capillary Endothelial Cells.*
Chemical Biology & Drug Design, 79: 507-513.
Impact Factor: **2.527**

Tóth, G.; **Mallareddy, J. R**.; Tóth, F.; Lipkowski, A. W.; Tourwé, D. *Radiotracers, Tritium Labelling of Neuropeptides.*
Arkivoc, (v): 163-174.
Impact Factor: **1.096**

Sum of impact factor: **8.83**

11.2. List of not related to the thesis publications

2012:

Németh, K.; **Mallareddy, J. R.;** Domonkos, C.; Visy, J.; Géza, T.; Péter, A. *Stereoselective Analysis of Tetrapeptide Diastereomers: Resolution of Biologically Active Endomorphin Analogues by Capillary Electrophoresis using Cyclodextrins.*
Journal of Pharmaceutical and Biomedical Analysis (under review)

Kovács, G.; Petrovszki, Z.; **Mallareddy, J. R**.; Tóth, G.; Benedek, G.; Horváth, G. *Characterization of Antinociceptive Potency of Endomorphin-2 Derivatives with Unnatural Amino Acids.*
Acta Physiologica Hungarica (in print)
Impact Factor: **1.226**

2011:

Vandormael, B.; Wachter, R. D.; Martins, J. C.; Hendrickx, P. M. S.; Keresztes, A.; Ballet, S.; **Mallareddy**, **J. R.**; Tóth, F.; Tóth, G.; Tourwé, D. *Asymmetric Synthesis and Conformational Analysis by NMR and MD of Aba- and α-MeAba-Containing Dermorphin Analogs.*
ChemMedChem, 6: 2035-2047.
Impact Factor: **3.306**

Sum of impact factor: **4.532**

11.3. Manuscripts under preparation

Borics, A.; **Mallareddy, J. R.**; Tímári, I.; Kövér, K. E.; Keresztes, A.; Tóth, G. *The Effect of Pro^2 Modifications on the Structural and Pharmacological Properties of Endomorohin-2.*

Tóth, F.; Kleczkowska, P.; Lipkowski, A. W.; **Mallareddy, J. R.;** Tourwé, D.; Tóth, G.; Bojnik, E.; Benyhe, S. *Synthesis and Binding Characteristics of a Neurotensin-like Peptide: [^{3}H]Neuromedin-N.*

11.4. Oral presentations

2009:

Mallareddy, **J. R.**; Keresztes, A.; Tóth, G "Investigations of Endomorphin-2 Biosynthesis" in Annual Meeting of the Peptide Committee of the Hungarian Academy of Sciences, **Balatonszemes**, Hungary.

2010:

Mallareddy, **J. R**. "Design, Synthesis and Biological Evaluation of Chemically Multiple Modified Endomorphins" in **Young Organic Chemist** symposium organized by Department of Chemistry, University of Szeged, Szeged, Hungary.

2011:

Mallareddy, **J. R.**; Borics, A.; Keresztes, A.; Tóth, G. "Design and Synthesis of Pharmacologically Active Endomorphins" in Annual Meeting of the Peptide Committee of the Hungarian Academy of Sciences, **Balatonszemes**, Hungary.

Mallareddy, **J. R**. "Effects of Unnatural Amino Acids on Bioactivity of Endomorphins" in **Young Organic Chemist** symposium organized by Department of Chemistry, University of Szeged, Szeged, Hungary.

11.5. Posters

2009:

Mallareddy, J. R.; Csibrany, B.; Keresztes, A.; Tóth, G. *From Precursor to Peptide*: *Evidence for endomorphin-2 Biosyntheis?*. 12th Meeting of the Hungarian Neuroscience Society, Budapest, Hungary. (**2009**)

2010:

Mallareddy, **J. R.**; Borics, A.; Keresztes, A.; Tóth, G. *Design, Synthesis and Pharmacological Evaluation of Novel Endomorphin analogues with Multiple Structural Modifications*. 8th European Opioid Conference, Krakow, Poland. PS I - 7

Tóth, G.; **Mallareddy**, **J. R**.; Borics, A.; Kövér, K. E.; Keresztes, A. *Design, Synthesis and Biological Evaluation of Novel Endomorphins with Multiple Structural Modifications*. Proceedings of 31st European Peptide Symposium, European Peptide Society, Copenhagen, Denmark. Page 454-455.

Tomczyszyn, A.; Lipkowski, A. W.; Toth, G.; Keresztes, A.; **Mallareddy, J. R.;** Misicka, A. *Synthesis of Tritiated Ligand for Binding Assays to Tachykinin Receptors*. 20th Polish Peptide Symposium, Wladyslawowo, Poland.

2011:

Mallareddy, **J. R**.; Borics, A.; Keresztes, A.; Tóth, G. *Influence of Proline Mimetics on Bioactivity of Endomorphin-2*. 4th European Conference on Chemistry for Life Sciences, Budapest, Hungary. P170

Tóth, G.; **Mallareddy, J. R.;** Tóth, F.; Lipkowski, A. W.; Tourwé, D. *Radiotracers-Tritium Labelled Neuropeptides*. 4th European Conference on Chemistry for Life Sciences, Budapest, Hungary. P175

Tóth, F.; Kleczkowska, P.; Lipkowski, A. W.; **Mallareddy**, **J. R**.; Tóth, G.; Tomboly, Cs.; Bojnik, E.; Borsodi, A.; Benyhe, S. *Synthesis and Binding Characteristics of a Neurotensin-like Peptide: [^{3}H]Neuromedin N*. International Society for Neurochemistry, Athens, Greece. (**2011**)