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

Thesis of Ph.D. Dissertation

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Szeged
2012

1. Introduction

Opioids have been the basis of pain treatment for thousands of years, and they remain so today. 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 pain relieving effect of opium and later of pure morphine has been successfully used against severe pain. 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.

Opioid peptides exert their effects through opioid receptors. Opioid receptors are members of the superfamily of G-protein-coupled receptors (GPCR). Three classical types of opioid receptors have been identified based on pharmacological and behavioral observations, namely μ-, δ-, and κ-receptors (MOR, DOR and KOR, respectively). Among those, μ-opioid receptors are targeted in the search for new drugs to suppress chronic pain. A major goal in opioid peptide research is the development of novel analgesics that could substitute morphine with less side-effects such as dependence, tolerance, respiratory depression and reward-seeking behavior. Endomorphin-1 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2) were initially isolated from bovine and later from human brain cortex. These peptides are the putative endogenous ligands of the μ-opioid receptor based on their high affinity and exceptional selectivity. Therefore they became important models in analgesic research. Its widely accepted that analgesic effects occur within the central nervous system (CNS) thus peptides should be able to cross the blood-brain barrier (BBB) intact. However, exogenous application of endomorphins is limited by short duration of action, low in vivo efficacy, poor metabolic stability and inability to cross the BBB. Even though the Endomorphins have been shown to have the longest half-lives among all endogenous opioid ligands, for their consideration as valuable therapeutic drugs it is essential to enhance their ability to enter the CNS and their resistance to enzymatic degradation. Such objectives may possibly be achieved through systematic modification of the peptide sequence.

2. Aims of the work

The main goal of our research is to develop endomorphin analogues in order to accomplish increased proteolytic stabilities while retaining or enhancing biological properties. To achieve aforementioned objectives we incorporated several unnatural amino acids into the structure of Endomorphins. 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 the 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 $pFPhe^4$ unnatural amino acids.
- ✓ To investigate the opioid receptor binding affinities and selectivities of newly synthesized analogues in radioligand receptor-binding studies.
- ✓ To test the functional properties of the highly potent analogues (selected based on receptor-binding assays results) in [^{35}S]GTPγS binding assay.
- ✓ To evaluate the enzymatic resistance of highly potent analogues (selected 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 characterize blood-brain-barrier permeabilities using tritiated endomorphin-2 analogues.
- ✓ To test the analgesic properties of highly potent endomorphin-2 analogues in *in vivo* chronic joint pain model.

3. Methods

3.1. Peptide synthesis and purification

A pool of endomorphin analogues were synthesized by manual solid phase peptide synthesis using N-α-t-Boc-protected amino acids and 4-methylbenzhydrylamine resin to obtain C-terminal amides. Obtained crude peptides were analyzed and purified using RP-HPLC on C_{18} column operating at 216 nm. Purity of peptides was determined by analytical RP-HPLC. The molecular weights of the peptides were confirmed by HRMS/ESI-MS.

3.2. Radioligand binding assay

The μ-receptor affinities were measured with [^{3}H]DAMGO (1 nM, 25^{o}C, 1 h, GF/C filter, glass tubes), and [^{3}H]Ile^{5,6}-deltorphin-2 (2 nM, 35^{o}C, 45 min., GF/B filter, plastic tubes) was used for δ-receptor affinities.

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

Ligand functional properties i.e. agonist or antagonist were measured based on efficacy (E_{max}) value. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS.

3.4. Enzymatic degradation

Evaluated ligand stabilities against proteolytic enzymes such as dipeptidyl peptidase IV, carboxypeptidase Y, amino peptidase etc..

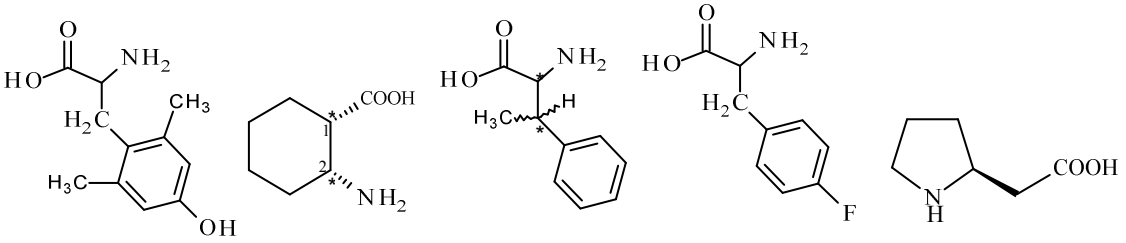
All of the biological assays (methods 3.2 - 3.4) were performed using rat brain membrane preparation.

3.5. Determination of n-octanol/water partition coefficient

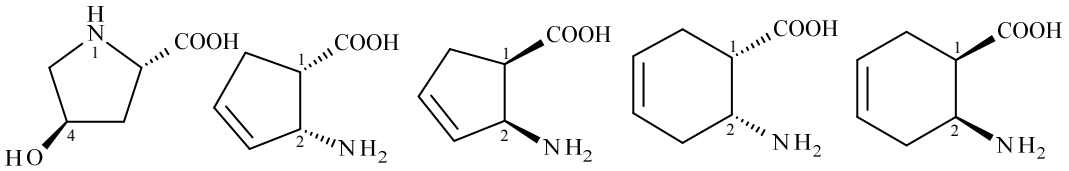
Characterized the hydrophobicity of the ligands expressed as the ratio of peptide concentration found in the octanol phase to that found in the aqueous phase.

4. Results and Discussion

In general, endomorphins are proteolytically less stable, although having the longest half-lives among the known mammalian opioid peptides. 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 incorporated 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), (2*S*,3*S* and 2*R*,3*R*)-β-methylphyenylalanine[4] (eβMePhe) and para-fluoro-phenylalanine[4] (pFPhe) into the sequence of endomorphins. The chemical structures of incorporated unnatural amino acids were shown below.



Dmt[1] *cis*-(1*S*,2*R*)/(1*R*,2*S*)Achc[2] (2S,3S)/(2R,3R)βMePhe[4] pFPhe[4] βPro[2]



Hyp[2] (1*S*,2*R*)ΔAcpc[2] (1*R*,2*S*)ΔAcpc[2] (1*S*,2*R*)ΔAchc[2] (1*R*,2*S*)ΔAchc[2]

The analysis of the binding data 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 in comparison with native peptides. These binding affinities were further enhanced by the combined substitution of Dmt[1], (1*S*,2*R*)Achc[2] and (2*S*,3*S*)βMePhe[4]. 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 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. 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. Furthermore, it is also interesting to mention that pFPhe^4 could compensate the detrimental effects of (1*R*,2*S*)Achc^2 incorporation. Substitution of Hyp^2 resulted in decreased affinity to μ-opioid receptor, in contrast to affinity values measured with opioid selective antagonist naloxone in crude rat brain membrane. Insertion of (*S*)-βPro^2 resulted in lower affinity compared to that of parent endomorphin-2. βPro^2 containing endomorphin-1 exhibited higher affinity to μ-opioid receptor in rat brain homogenate compared to the endomorphin-2. It can be assumed that only minor differences in structures (Trp/Phe) may be responsible for the observed changes in ligand binding. These results indicate that both the placement 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 MOR activity of EM-2. The observed propensity of binding potencies for unsaturated alicyclic β-amino acid containing analogues was similar to data obtained with saturated alicyclic β-amino acid analogues. Each ligand showed low to moderate binding affinities for δ-opioid receptors, indicates that these modifications provide μ-opioid receptor ligands.

On the basis of the heterolog displacement binding data, the most potent analogues were selected for [^{35}S]GTPγS functional assays to evaluate agonist/antagonist properties. To confirm opioid receptor functionality, the newly synthesized ligands were also assayed in the presence of the opioid antagonist naloxone. Potency (EC_{50}) and efficacy (E_{max}) values were compared with those of the μ-receptor full agonist DAMGO (E_{max}=178%). Dose-dependent increases were observed for selected compounds in [^{35}S]GTPγS binding. Both parent ligands exhibited comparable potencies, but lower efficacies (E_{max}=166% for endomorphin-1 and E_{max}=163% for endomorphin-2) was observed as compared to DAMGO, confirming that endomorphins are partial agonists, as reported earlier. Some of the tested compounds displayed the higher efficacies when compared to DAMGO, acting as full agonists. A few of compounds exhibited slightly less efficacies than that of DAMGO, which indicate partial agonist properties. No

significance difference was observed in efficacies measured with unsaturated alicyclic β-amino acid containing analogues when compared to that of saturated alicyclic β-amino acid containing analogues. Analogue containing Dmt[1], (1*S*,2*R*)Achc[2], pFPhe[4] displayed highest affinity and efficacy among all compounds. Incorporation of Hyp[2] revealed moderate efficacy (E_{max}=148%), suggesting that it is a moderate partial agonist. The lowest efficacy (E_{max}=116%) was observed for analogue containing βPro[2] among all analogues, proposing as a weak agonist. In the case of endomorphin-1, insertion of βPro[2] yielded the peptide with an increased efficacy, acting as an agonist. Interestingly, some compounds stimulated G-protein binding even in the presence of naloxone (10^{-5} M) therefore, we hypothesized that these ligands may bind to other G-protein coupled receptors or to non-opioid binding site.

On the basis of the competition binding experiments, most potent analogues were selected for enzymatic degradation studies. Those analogues containing alicyclic β-amino acids demonstrated prolonged half-lives (>20 h) relative to the endomorphins ($t_{1/2}$=5-7 min), proving the enzymatic resistance of the new analogues. These results confirmed that Pro[2] targeting modifications yields proteolytically stable endomorphins.

The BBB plays a significant role in the treatment of CNS disorders. The rate of transendothelial transport of the peptides from the luminal (blood) side to the abluminal (brain) side was characterized. All of the tested analogues showed a significantly increased permeability in comparison with parent endomorphin-2. None of the peptides affected the viability of the cells significantly, not even in the highest concentrations, suggesting no cytotoxic effect was achieved.

Based on the binding potencies, high potent endomorphin-2 analogues were selected to investigate the anti-allodynic effects at spinal level in a chronic joint pain model. All of the analogues elicited an antinociceptive effect in a concentration dependent manner.

5. Summary

- ✓ 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 analogues with increased proteolytic stabilities 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 relative to 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 endomorphin-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 endomorphin-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 endomorphin-2.
- ✓ Dmt[1], (1*S*,2*R*)Acpc[2], and (1*S*,2*R*)Achc[2] containing endomorphin-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.
- ✓ Among all the tested ligands Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2 elicited antinociception comparable with morphine in a chronic pain model.

List of thesis related publications

2011:

1. 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:

2. 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**

3. 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**

List of not related to the thesis publications

2012:

1. 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)

2. 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:

3. 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**

Manuscripts under preparation

1. 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.

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.

Oral presentations

1. **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. (**2011**)

2. **Mallareddy**, **J. R**. "Effects of Unnatural Amino Acids on Bioactivity of Endomorphins" in **Young Organic Chemist** symposium organized by University of Szeged, Szeged, Hungary. (**2011**)
3. **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. (**2010**)
4. **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**)

Posters

1. **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. (**2011**) P170
2. 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**)
3. 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. (**2011**) P175
4. **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. (**2011**) PS I – 7
5. 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. (**2010**)

6. 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. (**2010**)

Declaration of co-author renunciation

Undersigned, I declare that the candidate's thesis and the jointly published papers in which the candidate's role is of key of important weren't used and will never be used in the future for obtaining scientific degree.

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.* Chem. Biol. Drug Des. **2012**, *79*, 507-513.

Tóth, G.; **Mallareddy, J. R**.; Tóth, F.; Lipkowski, A. W.; Tourwé, D. *Radiotracers, Tritium Labelling of Neuropeptides.* Arkivoc, **2012**, *(v)*, 163-174.

Fazakas Csilla ……

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Dr. Imola Wilhelm ……

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