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Summary of Ph.D. Thesis

**Receptor binding and functional biochemical studies on
synthetic μ -opioid receptor selective ligands**

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INTRODUCTION

Opioid analgesics are of inestimable value because they reduce or abolish certain pain without causing a loss of consciousness, relieving coughs, spasms, fevers and diarrhea. Opioid drugs and endogenous opioids act through multiple opioid receptors. To date, there are three well-defined or "classical" types of opioid receptors: MOP (μ), KOP (κ), DOP (δ). These receptors are widely distributed throughout the brain and periphery. Their activation by ligands results in a multitude of effects. In general, μ - or δ -receptors selective agonists are analgesic and rewarding, κ -receptor selective agonists are dysphoric.

Opioid receptors belong to the membrane-bound class A (Rhodopsin) family of G/G_o protein-coupled receptors composed of a single polypeptide chain with an extracellular N-terminal domain, 7TM helical domains connected by three extracellular and three intracellular loops and an intracellular C-terminal tail. Opioid 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. The third intracellular loop has been implicated in the binding of G-proteins, and the high homology at this portion of the receptor suggests that various opioid receptors interact with similar G-protein complexes.

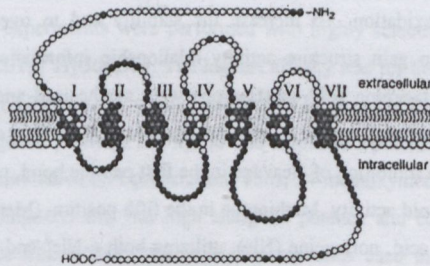


Fig.1. Schematic representation of an opioid receptor.

The binding of opioid agonists to the receptor triggers a chain of signaling events, which are involved in the cellular mechanisms of pain control and drug addiction.

Opioid Ligands

Met-enkephalin-Arg⁶-Phe⁷ (Tyr-Gly-Gly-Phe-Met-Arg-Phe; MERF) is a naturally occurring heptapeptide derived from the precursor polypeptide proenkephalin A. MERF is localized at the C-terminus within the prepropeptide structure and its sequence is well conserved among mammalian species. It was first isolated from bovine adrenal medullary granules and striatal extracts. Antinociceptive and antitussive effects of the peptide have been reported in mice, all of which were sensitive to naloxone antagonism, indicating that MERF exerts its pharmacological action through opioid receptors. MERF has also produced cardiovascular effects, and it seemed to be involved in immunity. Opioid receptor binding properties of MERF have been reported in various species using radiolabeled alkaloid compounds [³H]etorphine and [³H]ethylketocyclazocine. From these experiments it was concluded that the heptapeptide binds preferably to κ_2 -opioid receptors. The tritiated analogue of MERF was prepared in our laboratory and it was shown to label opioid (κ_2 and δ) sites in rat and frog brain membrane preparations and it also bound to non-opioid sites in rat and guinea-pig cerebellum and forebrain. Pentapeptide- and C terminally elongated enkephalins were reported to be sensitive to enzymatic degradation by peptidases, so selective inhibitors should be added to prevent decomposition of the peptide ligands. Methionine in the fifth position can also be susceptible to oxidation. To increase the stability and to overcome the problem of oxidation and to gain structure-activity relationship information, several new MERF analogues with D-amino acid substitutions were synthesized and tested in radioligand-binding assays as well as in *in vivo* pharmacological tests. Replacement of Gly² for D-Ala² results in inhibition of cleavage in the first peptide bond, protecting Tyr¹, which is essential for opioid activity. Methionine in the fifth position (Met⁵) was replaced with its isosteric amino acid, norleucine (Nle), utilizing both L-Nle⁵ and D-Nle⁵ substitutions. A novel analogue containing double D-amino acid substitutions at the second and fifth position, **Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe**, abbreviated as DADN exhibited changes in ligand-binding pattern when compared to the parent heptapeptide. In binding assays

using [^3H]MERF and [^3H]naloxone, lower affinities were observed for DADN compared to the natural structure. On the other hand, the new ligand showed substantially higher affinity toward μ -binding sites as measured by [^3H]DAMGO.

A derivative of the 14-alkoxymorphinan series, **14-methoxymetopon** (4,5 α -epoxy-3-hydroxy-14 β -methoxy-5 β ,17-dimethylmorphinan-6-one) appears to be a potent and selective-agonist, which exhibits an analgesic action far more potent than morphine (300-20,000-fold, depending upon the assay used) in diverse nociceptive tests (tail flick, hot plate, acetic acid-induced abdominal constriction test) in rats and mice. The dependence liability of the compound in the withdrawal-jumping test is less pronounced than that of morphine in rats and mice. Compared with sufentanil, 14-methoxymetopon elicits a slight respiratory depression, it does not induce hypoxia and hypercarbia, it produces less hypotension, bradycardia and it produces less sedative effects in the dog. 14-Methoxymetopon has been reported as being far more effective than morphine in reducing the emotive/affective component of pain and in producing an anxiolytic effect. In addition, it increased the serum corticosterone levels and the hypothalamic serotonin content. The enhanced potency of 14-methoxymetopon is increased with either spinal or supraspinal administration, where its analgesic activity is more than a million-fold greater than morphine. It showed approximately 3-fold greater affinity for μ_1 -opioid binding sites than μ_2 -opioid sites in competition binding assays performed in calf thalamus membrane preparations. The analgesic effect of the ligand was blocked only by μ -opioid receptor selective antagonists (naloxonazine and β -funaltrexamine). To establish the specificity of the ligand competition experiments were performed with highly selective radioligands: [^3H]DAMGO, [^3H]DSLET, [^3H]U69,593. The highest affinity was for the μ -sites. The *in vitro* agonist property of 14-methoxymetopon was proven by showing a sodium index of 41 that indicates high agonist potency. The ligand also exhibited a potent agonistic action on isolated electrically stimulated GPI preparation. Thus, 14-methoxymetopon appears to be a very promising compound that has high analgesic potency and considerably less pronounced adverse side effects than other opioid compounds used in therapy at the moment.

OBJECTIVES

Despite the big progress made in opioid research, further studies are needed to understand the mechanisms of action of opioid receptors. The development of new, highly selective ligands and investigation of the reasons underlying their selectivity is one of the ways leading to the full comprehension of the problem. μ -Opioids represent the major class of strong analgesics used clinically. The present study was dedicated to biochemical and functional analysis of opioid ligands acting at the μ -opioid receptor. The studied ligands include synthetic (heterocyclic) and modified endogenous compounds (peptide).

In the first part [^3H]Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe, an analogue of the naturally occurring Met-enkephalin-Arg⁶-Phe⁷ was biochemically analyzed. The main goals were:

- To have a novel opioid ligand with improved stability and specificity
- To measure its opioid activity in kinetic, equilibrium and competition binding studies
- To compare its opioid binding properties with those of other well-known compounds labeling opioid receptors.
- To test if this compound is able to generate the intracellular response characteristic to opioids (G protein activation).
- To test if this effect is μ -opioid receptor selective (inhibition by μ -antagonists).
- Determination of the agonist/antagonist character of the compound.

In the second part a derivative of the 14-alkoxymorphinan series, 14-methoxymetopon was analyzed functionally. The main goals were:

- To test if this compound is able to generate the intracellular response characteristic to opioids (G protein activation).
- To test if this effect is μ -opioid receptor selective (inhibition by μ -antagonists).
- Determination of the agonist/antagonist character of the compound.

METHODS

Membrane preparations

Preparation of rat brain membranes

A crude membrane fraction was prepared from Wistar rat forebrains according to a method of Pasternak with small modifications. Two-three month-old animals were decapitated, and the brains without cerebella were rapidly removed, and washed several times with chilled 50 mM Tris-HCl buffer (pH 7.4). The brains were weighed and suspended in 5 vol/wt of brain tissue of the ice-cold buffer. Tissues were homogenized using a Braun teflon-glass homogenizer (1000 rev./min, 10-15 strokes), and filtered through four layers of gauze to remove large aggregates. Additional buffer was added to reach a final buffer volume/membrane pellet ratio of 30 (ml/g). After centrifugation with a Sorvall RC5C centrifuge (40000x g, at 4°C, for 20 min), the resulting pellet was resuspended in fresh buffer (30 vol/wt) by using a vortex. The suspension was incubated at 37°C for 30 min to remove any endogenous opioids. Centrifugation was repeated under the same conditions as described above, and the final pellet was resuspended in 5 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose to give a final protein concentration of 3-4 mg/ml. The membrane samples were stored in 5 ml aliquots at -70°C. The protein concentration was determined by the Bradford method. Before use the membranes were thawed and resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged (40000x g, 4°C, 20 min) to remove sucrose and used immediately.

Membrane preparation from CHO δ , CHO κ and CHO μ cell cultures

Chinese hamster ovary (CHO) cells stably transfected with μ -, δ - or κ -opioid receptors (CHO μ , CHO δ and CHO κ cells) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) and in α -minimum essential medium (α MEM, Gibco), respectively. Both media were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 25 mg/ml fungizone and 0.5 mg/ml geneticin. Cells were maintained in culture at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air. Membranes were prepared from subconfluent cultures. Cells were rinsed three times with 10 ml PBS and removed with 50 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA and 0.1 mM PMSF buffer and homogenized for 15 sec with a polytron homogeniser in an ice-bath. Homogenates were centrifuged two times at 18,000xg for 20 min. The final pellet was resuspended in the above buffer containing 0.3 M sucrose and stored in aliquots at -70°C until use.

Receptor binding assay

The membrane suspensions from rat brain were incubated in polypropylene assay tubes for 30 min at 24°C with the radioligand in a final volume of 1 ml. Incubations were carried out in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 0.1 mM PMSF, 20 μ g/ml bacitracin, 40 KIU/ml trasylol and 1 mg/ml protease free BSA. Incubation was started by the addition of membrane suspension and terminated by rapid filtration through Whatman GF/C glass fiber filters presoaked in 0.3% PEI at pH 10, using a Brandel M24R Cell Harvester. After three washings with 5-ml portions of ice-cold buffer (50 mM Tris-HCl, 5 mM KCl, 2 mM MgCl₂, 0.1 mg/ml BSA, pH 7.4) filters were dried for 3 h at 37°C. The radioactivity was measured in a toluene-based scintillation cocktail, using a Packard TriCarb 2300TR spectrophotometer with 58% counting efficiency. Potencies of competing ligands were determined by co-incubation with 10⁻¹¹-10⁻⁴ M freshly prepared solutions of the unlabeled drugs with 0.5-1 nM tritiated ligand. Non-specific binding was defined as the radioactivity bound in the presence of 10 μ M unlabeled DADN or naloxone. All assays were performed in duplicate and repeated several times. Experimental data were analyzed by the GraFit, a non-linear least-squares curve fitting

programme or with GraphPad Prism. Data are generally expressed as arithmetic means \pm SEM of at least three repeated assays.

Determination of protein concentration:

The protein concentration was determined by the method of Bradford.

GTP γ S binding assay

Rat brain membranes (~ 10 μ g of protein/ tube) were incubated for 60 min at 30°C in Tris-EGTA buffer (50 mM Tris-HCl buffer, 3 mM MgCl₂, 1 mM EGTA, 100 nM NaCl, pH 7.4) containing 0.05 nM [³⁵S]GTP γ S with increasing concentrations (10^{-10} - 10^{-5} M) of opioid ligands tested in the presence of 30 μ M GDP in a final volume of 1ml. Basal activity was determined by subtracting the non-specific binding (measured in the presence of 100 μ M unlabeled GTP γ S) from the total binding (measured in the absence of tested compounds). The incubation was started by the addition of [³⁵S]GTP γ S and was terminated by vacuum filtration through Whatman GF/B glass fibre filters. Filters were washed three times with ice-cold 50 mM Tris-HCl buffer (pH 7.4) and then dried. The bound radioactivity was measured in a toluene-based scintillation cocktail, using a Packard TriCarb 2300TR liquid scintillation counter. Stimulation is given as percentage of the basal activity. Data were calculated by fitting sigmoid dose-response curves using the GraphPadPrism program.

NEW RESULTS AND CONCLUSIONS

μ -Opioids remain the prototypic opiate analgesics. Despite their widespread utility, a number of serious side effects have led to attempts to generate molecules exhibiting a favourable dissociation between analgesic activity and the side effects. The present study focused on the biochemical and functional analysis of opioid ligands acting at the μ -opioid receptor. The studied ligands include 14-methoxymetopon and D-Ala²-D-Nle⁵-MERF. The main findings are the following:

BIOCHEMICAL CHARACTERIZATION OF [³H]D-Ala²-D-Nle⁵-MERF and FUNCTIONAL ANALYSIS OF 14-METHOXYMETOPON

1. Incorporation of D-Ala² and D-Nle⁵ into MERF resulted in better stability and improved analgesic potency of the compound.
2. Specific binding of [³H]DADN to rat brain membranes was of high affinity, reversible, saturable, and stereoselective. DADN exhibits preference toward μ -opioid-binding sites.
3. Kinetic experiments showed that the association occurred rapidly according to pseudo-first order kinetics. Ligand-induced dissociation of the radioprobe proceeded in a

monophasic manner. Equilibrium saturation experiments revealed that a single class of opioid-binding sites was labeled by this radioligand.

4. Heterologous competition assays with different opioid compounds revealed that the rank order of potency is: $\mu > \delta > \kappa$ for [^3H]DADN specific binding.
5. High-affinity binding interaction was obtained in spinal cord membrane preparation.
6. The rank order of the potency of DADN in displacing [^3H]DADN was $\mu > \delta > \kappa$ in the cloned receptor-system (CHO cells).
7. Both DADN and 14-methoxymetopon activated G-proteins in a concentration dependent manner. G-protein stimulation of DADN and 14-methoxymetopon could be prevented by naloxone. CTAP and cyprodime (μ -receptor selective antagonists) effectively inhibited the activation of G-proteins in the case of both ligands. The stimulatory action of DADN was only slightly reversed by the κ -selective antagonist norBNI and δ -selective antagonist TIPP and was not reversed by the κ -selective antagonist nor-BNI and δ -selective antagonist HS378 in the case of 14-methoxymetopon. DADN and 14-methoxymetopon are potent opioid agonists and their actions are mediated via the μ -receptor.

Integrity of the tyrosine moiety at the amino terminal of opioid peptides is an essential requirement for opioid activity. D-Amino acid substitutions at the second position can lead to enhanced activity of various opioid peptides, and increase the duration of their pharmacological effects. Structural modifications targeted also the thioether ($-\text{R-S-CH}_3$) group of the Met⁵ side chain, which was shown to be readily oxidized resulting in methioninesulfoxide and/or methionine sulfone. In opioid peptides with Tyr-Gly N-terminal dipeptide, substitution of Gly² with D-Ala² protects the peptide against aminopeptidases, so this replacement increases the ligand's *in vivo* and *in vitro* stability without decreasing the opioid activity. In our studies DADN showed higher affinity for μ -opiate-binding sites as measured with [^3H]DAMGO. The reason for this is likely due to the presence of D-Nle⁵ side chain in the heptapeptide backbone. It is well known that the incorporation of D-amino acids into helical structured oligopeptides causes a change in the 3D structure by destabilizing the α -helix. In heptapeptides the chance of the peptide folding into α -helical conformation is limited (but with a well-

defined set of interactions α -helix may form in these short peptides). It is assumed, that the active conformation of DADN, which forms near the extracellular surface of the receptor molecule, favours interactions with μ -opiate receptors above all. The high potency of DADN in *in vivo* tests can be ascribable by conformational and stability factors and also by differences in solubility, because we found this peptide to be the most lipid soluble among all MERF analogues synthesized. The pronounced hydrophobicity of the ligand can increase a tight interaction with the μ -binding site. The increased lipophilicity of DADN favours the transport of the ligand through the blood-brain barrier and might take part in the reported analgesic effect of the molecule. Changes in the receptor-type preference of the peptide, increased chemical and enzymatic stability, and higher hydrophobicity (lipid solubility) all account for the effectiveness of the compound obtained in biochemical and pharmacological assays.

Based on these findings, DADN is a potent opioid agonist and its action was demonstrated to be mediated via the μ -opioid receptor. Altogether, incorporation of D-alanine² and D-norleucine⁵ into the naturally occurring proenkephalin-derived heptapeptide MERF resulted in improved analgesic potency and changed opioid receptor selectivity of the compound. [³H]DADN recognized and labeled μ -opioid receptors in rat brain membrane preparations. Importantly, the binding of this radioligand fulfilled the criteria of reversibility, saturability, stereoselectivity, and low non-specific binding necessary for valuable radioligands. These properties of [³H]DADN combined with its satisfactory purity, stability, and high specific radioactivity (41 Ci/mmol), make this radioligand a very promising tool for analyzing the properties and function of the μ -opioid receptor.

The highly potent epoxymorphinan analogue, 14-methoxymetopon activated G-proteins by stimulating [³⁵S]GTP γ S binding to rat brain membranes in a concentration-dependent manner. These functional data confirm previous findings in guinea-pig ileum bioassay on the agonist character of 14-methoxymetopon. Stimulation of 14-methoxymetopon could be prevented by 10 μ M naloxone, indicating an opioid receptor-mediated action. Moreover, the reversibility of these effects was shown with antagonists (CTAP, cyprodime) acting selectively at the μ -opioid receptor. In contrast, the stimulatory action of 14-methoxymetopon and DAMGO was not reversed by the selective κ - (nor-BNI) and

δ - (HS378) opioid receptor antagonists. Based on these findings, 14-methoxymetopon is a potent opioid agonist and its action was demonstrated to be mediated via the μ -opioid receptor. 14-methoxymetopon has a high analgesic potency with exceptionally mild side effects compared to morphine. It is one of the few nonpeptide μ -opioid receptor agonists available in radiolabeled form up to now. All the properties mentioned above should make this ligand an important and useful tool in probing μ -opioid receptor mechanisms, as well as to promote a further understanding of the opioid system at the cellular and molecular level.

LIST OF PUBLICATIONS

This thesis is based upon the following publications:

- I. M. Spetea, F. Tóth, J. Schütz, F. Ötvös, G. Tóth, A. Borsodi and H. Schmidhammer (2003) [^3H]14-Methoxymetopon, a high affinity ligand for the μ -opioid receptor. *In vitro* receptor binding and [^{35}S]GTP γ S functional assays in rat brain. *Eur. J. Neurosci.* **18**: 290-295.
- II. F. Tóth, J. Farkas, G. Tóth, M. Wollemann, A. Borsodi, S. Benyhe (2003) Synthesis and binding characteristics of a novel enkephalin analogue, [^3H]Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe. *Peptides* **24**: 1433-1440.
- III. F. Tóth, Gy. Horváth, M. Szikszay, J. Farkas, G. Tóth, A. Borsodi and S. Benyhe (2004) Pharmacological and functional biochemical properties of D-Ala 2 -D-Nle 5 -enkephalin-Arg-Phe (DADN). *Regul. Pept.* **122**: 139-146.

Other publications

A. W. Lipkowski, P. Kosson, A. Misicka, D.B. Carr, V.J. Hruby, M. Yoshikawa, **F.Tóth**, G. Tóth (2003) Fragments of HIV GAG Polyproteins expressing affinity to opioid receptors. *Biopolymers* 71(3): 294.

Oral presentations at conferences

1. **Tóth F.** (2000) Egy szintetikus opioid heptapeptid, a Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe vizsgálata opioid receptor kötési tesztekben. Tudományos Diákköri Konferencia; Szeged, Hungary: April 20.

2. **Tóth F.** (2001) Biochemical characterization of a synthetic heptapeptide enkephalin analogue. Polish-Hungarian Ph.D. Meeting; Szeged, Hungary: October 24-26.

3. **F. Tóth**, J. Farkas, G. Tóth, S. Benyhe, A. Borsodi (2002) Binding and signalling properties of a synthetic enkephalin analogue. IBRO International Workshop on Signalling Mechanisms in the Central and Peripheral Nervous System; Debrecen, Hungary: January 24-26.

4. M. Wollemann, S. Benyhe, **F. Tóth** (2003) Nonopioid actions of opioid peptides. International Narcotics Research Conference (INRC); Perpignan, France: July 6-11.

5. Szemenyei E., **Tóth F.** Varga É., Jambrosic J., Yamamura H.I., Bilsky E., Borsodi A., Tóth G. (2004) 2', 6'-dimetil-Tyr¹ és eritro-β-metil-Phe⁴ endomorfín származékok. Peptidkémiai Munkabizottság Tudományos Ülése; Balatonszemes, Hungary: May 26-28.

6. Szemenyei E., **Tóth F.**, Varga É., Bilsky E., Tóth G.. (2005) Új nem természetes aminosavakat tartalmazó endomorfín analógok szintézise és biológiai jellemzése. Szegedi Ifjú Szerves Kémikusok 5. Tudományos Előadótalálkozója; Szeged, Hungary: January 12.

Poster presentations at conferences

1. Farkas J., Wollemann M., **Tóth F.**, Szikszay M., Benyhe S. and Tóth G. (1999) Novel peptide agonist ligands for the Met-enkephalin-Arg⁶-Phe⁷ binding sites: synthesis and binding properties. Italian-Hungarian-Polish Joint Meeting on Medicinal Chemistry; Catania, Italy, PB21 p. 201.
2. **Tóth F.**, Farkas J., Tóth G. and Benyhe S. (2000) Binding characteristics of the opioid heptapeptide D-Ala²-D-Nle⁵-enkephalin-Arg⁶-Phe⁷ (DADN) in rat brain membranes. European Neuropeptide Club "Neuropeptides 2000"; Innsbruck, Austria: May 8-13.
3. **Tóth F.**, Farkas J., Tóth G., Borsodi A. és Benyhe S. (2000) A D-Ala²-D-Nle⁵-enkephalin-Arg⁶-Phe⁷ (DADN) heptapeptid receptor szelektivitásának vizsgálata patkány agyban és μ - vagy κ -opioid receptorokat expresszáló sejtenyészetekben. Magyar Biokémiai Egyesület Molekuláris Biológiai Munkaértekezlet; Sopron, Hungary: May 8-11.
4. **Tóth F.**, Farkas J., Tóth G., Borsodi A. és Benyhe S. (2001) Egy szintetikus proenkefalin-A heptapeptid-analóg kötődési és jelátviteli tulajdonságainak vizsgálata patkány agyi membránpreparátumban. XXXI. Sümegi Membrán-Transzport Konferencián; Sümeg, Hungary: May 22-25.
5. **F. Tóth**, J. Farkas, G. Tóth, A. Borsodi and S. Benyhe (2002) Biochemical characterization of the opioid heptapeptide [³H]D-Ala²-D-Nle⁵-enkephalin-Arg⁶-Phe⁷ (DADN). 4th European Opioid Conference; Uppsala, Sweden: April 7-9.
6. H. Schmidhammer, F. Ötvös, J. Schütz, **F. Tóth**, G. Tóth, A. Borsodi, M. Spetea (2002) Biological characterization of [³H]14-methoxymetopon, a highly potent μ -opioid receptor agonist. 4th European Opioid Conference; Uppsala, Sweden: April 7-9.
7. **F. Tóth**, J. Farkas, G. Tóth, Gy. Horváth, M. Szikszay, A. Borsodi, S. Benyhe (2002) Biochemical, functional and pharmacological characterization of a synthetic MEAP

analogue. 33rd International Narcotics Research Conference; Asilomar Conference Center, Pacific Grove, California, USA: July 9-14.

8. Borsodi A., **Tóth F.**, Farkas J., Tóth G., Horváth G., Szikszay M., Benyhe S. (2002) Changes in opioid receptor specificity upon structural modification of Met-enkephalin-Arg-Phe. 3rd Forum of European Neuroscience; Paris, France: July 13-17.

9. **F. Tóth**, M. Spetea, J. Schütz, H. Schmidhammer, F. Ötvös, G. Tóth, A. Borsodi (2003) 14-Methoxymetopon, a novel powerful mu-opioid receptor agonist, [³⁵S]GTPgammaS functional assays in rat brain. Magyar Idegudományi Társaság (MITT); Balatonfüred, Hungary: January 22-25.

10. Szemenyei E., **Tóth F.**, Tóth G. (2003) Módosított szerkeztű és radioaktívan jelzett endomorfín származékok. Magyar Kémikusok Egyesülete (MKE), Vegyészkonferencia, Szerves és Gyógyszerkémia; Hajdúszoboszló, Hungary: June 26-28.

11. M. Spetea, **F. Tóth**, J. Schütz, F. Ötvös, G. Tóth, S. Benyhe, A. Borsodi, H. Schmidhammer (2003) Biological characterization of [³H]14-Methoxymetopon, a highly potent mu opioid receptor agonist. International Narcotics Research Conference (INRC); Perpignan, France: July 6-11.

12. **F. Tóth**, J. Farkas, G. Tóth, Gy. Horváth, M. Szikszay, A. Borsodi and S. Benyhe (2003) Biochemical, functional and pharmacological characterization of a synthetic opioid heptapeptide D-ALA²-D-NLE⁵-ENKEPHALIN-ARG⁶-PHE⁷ (DADN). Sixth IBRO World Congress OF Neuroscience; Prague, Czech Republic: July 10-15.

13. **F. Tóth**, M. Spetea, J. Schütz, H. Schmidhammer, F. Ötvös, G. Tóth, A. Borsodi (2003) Evaluation of receptor-mediated activation of regulatory G-proteins by 14-Methoxymetopon, a novel mu-opioid receptor agonist. Molecular Mechanisms in Signal Transduction EMBO/FEBS advanced lecture course; Spetses, Greece: August 18-29.

14. **F. Tóth**, J. Farkas, G. Tóth, A. Borsodi and S. Benyhe (2003) Synthesis, binding characteristics and G-protein activation of a novel synthetic Met-enkephalin-Arg-Phe analogue. Polish-Austrian-German-Hungarian-Italian Joint Meeting on Medicinal Chemistry; Kraków, Poland: October 15-18.

15. E. Szemenyei, **F. Tóth**, É. Varga, J. Jambrosic, E. Navratilova, H.I. Yamamura, A. Borsodi, G. Toth. New endomorphin analogues with 2',6'-dimethyl-Tyr and e- β -MePhe substitutions. (2004) 5th European Opioid Conference; Visegrád, Hungary: April 4-7.

16. **F. Tóth**, Gy. Horváth, M. Szikszay, J. Farkas, G. Tóth, A. Borsodi and S. Benyhe. Pharmacological and functional biochemical properties of D-Ala²-D-Nle⁵-enkephalin-Arg-Phe (DADN). (2004) Neuropeptides 2004, XIV European Neuropeptide Meeting; Alicante, Spain: May 9-12.

17. Cs. Tömböly, **F. Tóth**, G. Tóth, A. Borsodi, D. Tourwé. Endomorphin-2 analogues with a novel spirolactam type β -turn mimetic. (2004) 3rd International and 28th European Peptide Symposium; Prague, Czech Republic, September 5-10.

18. **F. Tóth**, E. Szemenyei, G. Tóth, É. Varga, J. Jambrosic, H. I. Yamamura, E. Bilsky, S. Benyhe, A. Borsodi Structurally modified novel endomorphin analogues with 2', 6' -dimethyl-Tyr and e- β -MePhe substitutions. (2005) Neuropeptides 2005, XV European Neuropeptide Meeting; Riga, Latvia: May 19-21.

19. E. Szemenyei, **F. Tóth**, É. Varga, J. Jambrosic, H. I. Yamamura, E. Bilsky, A. Borsodi, G. Tóth. New endomorphin analogues with 2',6'-dimethyl-Tyr and e- β -MePhe substitutions. (2005) Joint Meeting on Medicinal Chemistry; Vienna, Austria : June 20-23.