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Summary of the Ph.D. thesis

Biochemical, functional and pharmacological characterization of novel hexapeptide ligands targeting nociceptin/orphanin FQ receptor, *in vitro* and *in vivo* studies

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INTRODUCTION

The cloning of opioid receptors in the beginnings of 90's, was soon followed by the cloning of a novel **G-protein coupled receptor** that displayed very high homology with opioid receptors but did not bind opioid ligands. This receptor was then deorphanized by discovering its endogenous ligand the heptadecapeptide **nociceptin/orphanin FQ (N/OFQ).** This was the first successful example of the reverse pharmacology approach. The N/OFQ and its **N/OFQ peptide receptor (NOP)** are widely distributed in the central nervous system, but also in the periphery and in the immune system. Accordingly, N/OFQ elicits a broad range of biological effects such as modulation of pain transmission, of anxiety and response to stress, learning and memory, food intake, locomotor activity. In addition N/OFQ also controls some functions of the renal, cardiovascular, respiratory, and gastrointestinal. Thus, the NOP receptor likely represents an interesting molecular target for the development of novel therapeutics.

The following ligands are considered up to now the best pharmacological tools for investigating the consequences of NOP receptor selective activation or blockage: among NOP receptor agonists the peptide UFP-102 [(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2 and the non peptide Ro 64-6198 and among NOP receptor antagonists the peptide UFP-101 and the non peptides J-113397 and SB-612111. In addition to these compounds, in 1997 a series of hexapeptides of the general formula **Ac-RYY-R/K-W/I-R/K-NH_2** having high affinity and selectivity for the NOP receptor were identified from a synthetic combinatorial hexapeptide library containing about 52 million of compounds made considering all the natural amino acids except cystein. These hexapeptides were reported to behave as partial agonists at recombinant NOP receptors expressed in Chinese Hamster Ovary (CHO) cells (measured by [^{35}S]GTPγS binding and cAMP accumulation assays) and at native NOP receptor expressed in the mouse *vas deferens* (estimated by the inhibition of the twitch response induced by electrical field stimulation). These findings were later confirmed and extended by several research groups mainly using two of these hexapeptides namely Ac-RYYRIK-NH_2 and Ac-RYYRWK-NH_2, which were also used as chemical template for performing structure-activity studies. The head to tail cyclization of

Ac-RYYRWK-NH_2 produced a drastic decrease in binding affinity while the N-terminal acylation with a pentanoyl group or the replacement of the $Tyr^{2,3}$ residues with (pF)Phe led to the identification of high affinity low efficacy NOP receptor ligands. The N-terminal alkylation of the central core YYRW with groups bearing a guanidine function generated a NOP receptor agonist. Moreover the C-terminal addition of a polylysine sequence generated the peptide Ac-RYYRWKKKKKKK-NH_2, named ZP-120, which behaved similarly to the reference compound as a NOP receptor selective partial agonist but displaying higher affinity, metabolic stability and *in vivo* duration of action. Finally, we have prepared a high affinity NOP receptor ligand with antagonist properties by substituting the C-terminal amide with an alcoholic function, resulting **Ac-RYYRIK-ol**. This hexapeptide alcohol was further characterized by *in vitro* and *in vivo* approaches and turned out to be a low efficacy NOP receptor agonist with high potency, selectivity of action, metabolic stability and *in vivo* activity. Additionally, it was aimed to determine the structural requirements of the hexapeptide alcohol acting in the N/OFQ peptide – NOP receptor system. For reaching this goal structure-activity studies were done by systematic replacement of arginine with citrulline, and by modifying the N-terminal of the hexapeptide alcohol.

AIM OF STUDIES

Until now, relatively few ligands (agonists, antagonists and partial agonists) have been identified that have high NOP receptor selectivity and potency. Thus, novel ligands are still needed both as research tools for further investigations on the N/OFQ – NOP receptor systems and as drug prototypes to establish the therapeutic potential of drugs, that selectively interact with this receptor.

The present study was dedicated to biochemical, functional and pharmacological characterization of NOP receptor ligands in particular novel hexapeptide derivatives. This study includes the basic characterization of a novel synthetic hexapeptide by *in vitro* and *in vivo* analyses, and the structure-activity relationship studies performed on this compound. The main goals of the study were:

- To identify and characterize putative, selective, potent and stable NOP receptor ligands ***in vitro*** and ***in vivo***.
- To investigate the binding properties to the native receptors of **rat brain membranes** and to **recombinant human NOP receptors**
- To examine the post-binding effects (G-protein activation) in the functional biochemical **[^{35}S]GTPγS binding assay**.
- To examine the biological effects in the native receptors localized in N/OFQ sensitive tissues that are isolated from **mouse *vas deferens*** and **mouse colon**.
- To characterize the novel hexapeptide *in vivo* by i) **analgesiometric assay** such as tail withdrawal, to see its action on **supraspinal** and **spinal** level; ii) **spontaneous locomotor activity test;** iii) **food intake studies;** iv) **forced swimming test**.

EXPERIMENTAL PROCEDURE

Structural modifications on the hexapeptide template were performed by the research group of peptide chemistry led by Dr Anna Magyar Eötvös Loránd University, Budapest, Hungary. For the biochemical, functional and pharmacological characterization of the hexapeptides, a series of *in vitro* and *in vivo* experiments were performed. For the receptor binding and [^{35}S]GTPγS binding assay Wistar rat brain membranes and Chinese Hamster ovary cells expressing human NOP receptor (CHO-NOP$_h$) were used. CHO-NOP$_h$ cell lines were kindly provided by Dr Jean-Claude Meunier and Dr Maïthé Corbani from Toulouse, France. For the bioassays the tissues were isolated from Swiss mice and CD1/C57BL6/J-129 wild type (NOP$^{+/+}$) and NOP receptor knockout (NOP$^{-/-}$) mice.

For the *in vitro* characterization and the structure-activity studies:

- Radioligand binding experiments (Saturation, Displacement binding)
- [^{35}S]GTPγS binding assays
- Bioassays on isolated tissues (Mouse vas deferens, mouse colon bioassay) were performed

A series of *in vivo* assays were performed on mice injected with the hexapeptide alcohol, **Ac-RYYRIK-ol** intracerebroventricularly (i.c.v.) or intrathecally (i.t.).

For the *in vivo* characterization of the hexapeptide alcohol, **Ac-RYYRIK-ol**:

- Tail withdrawal assay
- Spontaneous locomotor activity test
- Food intake study
- Forced swimming test were carried out.

The experiments were performed as described in Kocsis et al, 2004, Regulatory Peptides, 122(3):199-207, Gunduz et al, 2006, NeuroSignals accepted Manuscript No: NS162-06, Gunduz et al, 2006, European Journal of Pharmacology, in press.

SUMMARY OF RESULTS

Following the discovery of the nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) and its endogenous ligand, an extensive search has started to find selective agonists and antagonists, targeting this novel receptor-ligand system, due to their therapeutic potentials. By the help of combinatorial chemistry a series of hexapeptides with a general formula of Ac-RYY-R/K-W/I-R/K-NH_2 were identified and found to have high NOP receptor affinity and selectivity. The present work was focused on the biochemical, functional and pharmacological characterization of novel NOP receptor ligands, **Ac-RYYRIK-ol** and analogues, which appear from the C-terminal modification of one of the previously reported hexapeptide. This work consists of basic characterization of Ac-RYYRIK-ol by *in vitro* and *in vivo* experiments and the structure-activity relationship studies performed on the novel hexapeptide.

The main findings are as follows:

1. C-terminal carboxyamide modification to a hydroxymethylene yielded high affinity (pK_i = 9.10 and 9.39 in rat brain membrane and CHO-NOP_h receptors, respectively), selective NOP receptor ligand, Ac-RYYRIK-ol.

2. N-terminal modifications with several acyl groups (ClAc-, pivaloyl-, formyl-, benzoyl-, mesyl-) decreased the affinity of the ligand towards the NOP receptor.

3. The replacement of the positively charged arginine (Arg) residues with an uncharged citrulline (Cit) clearly indicated that Arg at the first position is essential for NOP receptor binding and activity.

4. Ac-RYYRIK-ol (10 - 100 nM) competitively antagonized (pA_2 = 8.67) the N/OFQ induced G-protein activation in rat brain membranes. However, in CHO cells expressing NOP receptors with binding capacities (B_{max}) about two-times higher as in rat brain, the hexapeptide alcohol behaved as an agonist with high potency (pEC_{50} 8.52) and efficacy (E_{max} = 540.9 %).

5. Ac-RYYRIK-ol (10 - 1000 nM) competitively antagonized (pA_2 = 8.46), the inhibitory effect of N/OFQ in the electrically stimulated mouse *vas deferens*.

6. Ac-RYYRIK-ol mimicked the contractile effects of N/OFQ in the isolated mouse colon thus behaving as a NOP receptor agonist (pEC_{50} = 9.09). This effect was no longer evident in colon tissues taken from transgenic mice lacking the NOP receptor gene ($NOP^{-/-}$) indicating the NOP receptor selectivity.

7. In a series of in vivo experiments performed on Swiss mice, similarly to N/OFQ, Ac-RYYRIK-ol (dose range 0.001 - 1 nmol) was found to produce:
   i) pronociceptive effects after intracerebroventricular (i.c.v.) administration and antinociceptive actions when given intrathecally (i.t.) in the tail withdrawal assay;
   ii) inhibition of locomotor activity
   iii) stimulation of food intake after supraspinal administration. In the forced swimming test, Ac-RYYRIK-ol was inactive *per se*, but reversed the antidepressant-like effects elicited by the NOP receptor selective antagonist UFP-101 ([Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2).
   Thus in all *in vivo* assays Ac-RYYRIK-ol behaved as a potent NOP receptor agonist.

In conclusion, Ac-RYYRIK-ol displayed a complex pharmacological profile, which is likely due to the low efficacy agonist nature of this novel ligand of the NOP receptor. It is worthy of mentioning that NOP receptor partial agonists are drug candidates for management of hyponatremia and water retention (for indications such as aquaresis) in particularly because of their selectivity to produce renal, but not cardiovascular effects (Table 1). The high potency, selectivity of action, and *in vivo* effectiveness make Ac-RYYRIK-ol a useful pharmacological tool for future studies in the field of N/OFQ and its NOP receptor.

Table 1: Potential therapeutic applications of NOP receptor ligands.

AGONISTS	PARTIAL AGONISTS	ANTAGONISTS
Anxiolytics Stimulants of food intake Antitussives Anti-epileptics Spinal analgesics Suppressants of drug abuse For management of hyponatremia and water retention	Aquaretics (peripherally acting) for managements of hyponatremia and water retention	Analgesics (alone/in combination with opiates) Antidepressants Anorectics Nootropic agents Antiparkinson

LIST OF PUBLICATIONS

The publications related to the thesis:

1. **Gündüz, Ö.**, Sipos, F., Spagnolo; B., Kocsis, L., Magyar, A., Orosz, Gy., Borsodi, A., Calò, G., Benyhe, S. **(2006)**. In vitro binding and functional studies of Nociceptin / Orphanin receptor hexapeptides. *NeuroSignals*, accepted for publication. (Manuscript No: NS162-06) I.F. 3.585

2. **Gündüz, Ö.**, Rizzi, A., Baldisserotto, A., Guerrini, R., Spagnolo; B., Gavioli, E.C., Kocsis, L., Magyar, A., Benyhe, S., Borsodi, A., Calò, G. **(2006)**. In vitro and in vivo pharmacological characterization of the nociceptin/orphanin FQ receptor ligand Ac-RYYRIK-ol. *European Journal of Pharmacology*, in press [Epub ahead of print]. I.F. 2.432

3. Ligeti, M., Bősze,Sz., Csámpai, A., **Gündüz, Ö.**, Al-Khrasani, M., Rónai, A.Z., Medzihradszky-Schweiger H, Benyhe, S., Borsodi, A., Hudecz, F., Magyar, A. **(2006)**. Synthesis of enzymatically resistant Nociceptin-related peptides containing a carbamic acid residue. *Journal of Peptides Sciences*, in press [Epub ahead of print]. I.F. 1.652

4. Ligeti, M.*, **Gündüz, Ö.***, Magyar, A., Kató, E., Rónai, A.Z., Vita, C., Varga, I., Hudecz, F., Tóth, G., Borsodi, A., Benyhe, S. **(2005)**. Synthesis and Biological Studies of Nociceptin Derivatives Containing the DTPA Chelating Group For Further Labeling With Therapeutic Radionuclides, *Peptides*, 26(7):1159-1166. (*the first two authors have contributed equally to this work.) I.F. 2.511

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Proceedings:

1. **Gündüz, Ö.,** Ligeti, M., Magyar, A., Kató, E., Rónai, A.Z., Vita, C., Hudecz, F., Borsodi, A., Benyhe, S. A biological study on newly synthesized DTPA chelating group containing nociceptin derivative. **(2004)** *XIV European Neuropeptide Meeting*, Alicante, Spain. P2, published in Neuropeptides 38 (2004) 385-424. Page 415-416.
I.F. 2.494

2. Benyhe, S., **Gündüz, Ö.**, Farkas, J., Kocsis, L., Sipos, F., Ligeti, M., Magyar, A., Orosz, Gy., Tóth, G., Borsodi, A. Characterization of nociceptin binding sites by novel peptide analogs and radioprobes **(2003)**. *14th Meeting of the European Society for Neurochemistry*, Warsaw, Poland, Journal of Neurochemistry, 85(2):32.
I.F. 4.824

3. **Gündüz, Ö.**, Kocsis, L., Ligeti, M., Magyar, A., Orosz, Gy., Farkas, J., Tóth, G., Borsodi, A., Benyhe, S. Characterization of nociceptin receptor interaction with prepronociceptin peptides by newly developed unlabelled and tritium-labelled ligands. **(2002)**, in Proceedings of the closing seminar of the International training course 2001/02.

Oral presentations:

1. **Gündüz Ö.,** Rizzi, A., Gavioli, E.C., Spagnolo; B., Kocsis, L., Magyar, A., Benyhe, S., Borsodi, A., Calò, G. Hexapeptides targeting Nociceptin/Orphanin FQ Receptor: *in vitro* and *in vivo* Studies **(2006)** Ph.D. Days, University of Szeged, Szeged, Hungary.

2. **Gündüz, Ö.**, Kocsis, Ö., Sipos, F., Orosz, Gy., Magyar, A., Al-Khrasani, M., Kató, E., Rónai, A.Z., Farkas, J., Tóth, G., Borsodi, A., Benyhe, S. **(2004)** Searching for nociceptin antagonists among N-acetylated hexapeptide alcohols. *5th European Opioid Conference*, Visegrád, Hungary.

3. **Gündüz Ö.**, L. Kocsis, F. Sipos, Gy. Orosz, A. Magyar, M. Al-Khrasani, E. Kato, A.Z. Rónai, J. Farkas, G. Tóth, A. Borsodi, S. Benyhe. Recent developments in peptide antagonists targeting nociceptin receptor (NOP). **(2004)** Straub-Days, Biological Research Center, Szeged, Hungary.

4. **Gündüz, Ö.**, Kocsis, L., Ligeti, M., Magyar, A., Orosz, Gy., Farkas, J., Tóth, G., Borsodi, A., Benyhe, S. **(2002)** Characterization of nociceptin receptor interaction with prepronociceptin peptides by newly developed unlabelled and tritium-labelled ligands. Proceedings of the closing seminar of the International Training Course 2001/200 pp. 42-51.

Poster presentations:

1. **Gündüz, Ö,** Rizzi, A., Gavioli, E.C., Spagnolo; B., Kocsis, L., Magyar, A., Benyhe, S., Borsodi, A.; Calò, G. **(2006)** *In vitro* and *in vivo* pharmacological evaluation of Ac-RYYRIK-ol, a novel nociceptin/orphanin FQ receptor ligand. *6th European Opioid Conference*, Salamanca, Spain.

2. Benyhe, S, Sipos, F., Kocsis, L., Magyar, A, Borsodi, A., Spagnolo, B., Calò, G., **Gündüz, Ö. (2006)**. Ac-RYYRIK-NH_2 hexapeptide analogues targeting nociceptin (nop) receptor. *6th European Opioid Conference*, Salamanca, Spain.

3. **Gündüz, Ö.**, Rizzi, A., Gavioli, E.C., Spagnolo; B., Kocsis, L., Magyar, A., Benyhe, S., Borsodi, A., Calò, G. **(2006)** Pharmacological evaluation of Ac-RYYRIK-ol, a novel nociceptin/orphanin FQ receptor ligand: *in vitro* and *in vivo* studies. *International IBRO workshop*, Budapest, Hungary.

4. **Gündüz, Ö.**, Rizzi, A., Gavioli, E.C., Spagnolo; B., Kocsis, L., Magyar, A., Benyhe, S., Borsodi, A. Calò, G. **(2005)** *In vitro* and *in vivo* pharmacological characterization of the novel nociceptin/orphanin FQ receptor ligand Ac-RYYRIK-ol., *Society for*

Neurosciece (SfN) 35th annual meeting, November 12-16, 2005, Washington DC, USA. Poster number: 291.13.

5. **Gündüz, Ö.**, Kocsis, L., Sipos, F., Orosz, Gy., Magyar, A., Al-Khrasani, M., Kato, E., Rónai, A.Z., Farkas, J., Tóth, G., Borsodi, A., Benyhe, S. **(2005)** Ac-RYYRIK-ol, a novel potent hexapeptide for the nociceptin/orphanin FQ receptor: in vitro receptor binding and functional biochemical studies. *European Neuropeptide Club 2005, 15th Annual Meeting*, Riga, Latvia.

6. Apaydin, S., **Gündüz, Ö.**, Benyhe, S., Öktem, H.A. **(2004)** Modulation of opioid receptor binding by arachidonic acid. *5th European Opioid Conference,* Visegrád, Hungary. P4.

7. Ligeti, M., Magyar, A., Kató, E., Rónai, A.Z., **Gündüz, Ö.**, Benyhe, S., Vita, C., Hudecz, F. **(2004)** Synthesis and biological studies of nociceptin derivative containing DTPA chelating group. *5th European Opioid Conference,* Visegrád, Hungary. P29.

8. Benyhe, S., **Gündüz, Ö.**, Sipos, F., Kocsis, L., Ligeti, M., Magyar, A., Orosz, Gy., Farkas, J., Tóth G., Borsodi, A. **(2004)** Novel Synthetic Peptide Ligands Targeting The Nociceptin Receptor. *Conference on International Pharmacotherapy*, Brisbane Australia.

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11. Benyhe, S., **Gündüz, Ö.**, Kocsis, L., Ligeti, M., Magyar, A., Orosz, Gy., Al-Khrasani, M., Ronai, A.Z., Tóth, G., and Borsodi, A. **(2002)** Receptor binding and functional studies on novel synthetic nociceptin peptide analogs. *Proceedings of the International Narcotic Research Conference*, Asimolar Conference Centre, Pacific Grove, California, USA.

12. Benyhe, S., **Gündüz, Ö.**, Kocsis, L., Ligeti, M., Magyar, A., Orosz, Gy., Tóth, G., and Borsodi, A. **(2002)** Nociceptin analogs: Binding and functional properties. *4th European Opioid Conference*, Uppsala, Sweden

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