PhD thesis booklet

Biochemical, functional and pharmacological characterization of novel bifunctional peptide ligands and nociceptin variants

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INTRODUCTION

In 1994, following the discovery of the μ (MOP), δ (DOP), and κ (KOP) opioid receptor structures by molecular cloning, another similar receptor was identified and termed ORL1 (opioid receptor like 1), a so-called 'orphan' receptor with an unknown ligand partner. Deorphanisation of ORL1 receptor proceeded parallelly by two research teams. Around the same time in 1995 both groups identified a heptadecapeptide as primary endogenous ligand for the orphan ORL1 receptor. The identical peptides were independently named as orphanin-FQ and nociceptin, respectively. These important studies are now considered pioneers of the so-called 'reverse pharmacology' approach for drug discovery. The suggested nomenclature for the peptide involves the full name 'nociceptin' and the 'N/OFQ' abbreviation. The proper names for the receptor are 'nociception receptor' or 'NOP receptor' or 'NOPr', with the meaning of 'Nociceptin Opioid Peptide receptor'.

The NOP receptor belongs to the superfamily of G-protein coupled receptors (GPCRs) and to the family of rhodopsin-like receptors. Activation of the NOP receptor is mediated via G_i/G_o proteins. Comparing the cDNAs of the NOP receptor, classical opioid receptors and other GPCRs, it is apparent that they contain a number of conserved amino acids and motifs. The TM2, TM3 and TM7 domains of opioid receptors show significant (70%) conservation. Intracellular loops (ICL) are also greatly homologous, particularly ICL3 (>80%), which plays a role in G protein activation.

Regarding the tertiary structure of the opioid receptors, it has been found that the ligand binding pocket is located within the transmembrane helices in case of all four receptors. The message domains of endogenous opioid peptides (YGGF and FGGF) bind to this site.

Although the structures of NOP and classical opioid receptors are closely related, the morphine-based general opioid antagonist naloxone is unable to inhibit the NOPr mediated effects. In addition, the NOP receptor binds opioid ligands very weakly at most. The tetrapeptide segment at the N-terminus of the endogenous opioid peptide ligands, called the message domain, is responsible for this high selectivity. According to the Schwyzer's hypothesis, the message domain determines whether a peptide binds to classical opioid receptors or not, whereas the address domain determines the MOP, DOP or KOP receptor preference/selecivity of the peptide. This also explains why nociceptin cannot or very poorly

bind to classical opioid receptors, since its message domain differs from the opioid message domain.

The NOP receptor and its ligand are present not only in the central nervous system but also in many peripheral organs and even in the immune system. The N/OFQ-NOP receptor system plays an important role in the cardiovascular and renal system. Moreover, it has been described that it inhibits gastrointestinal and airway motility. It also behaves as an immunomodulator, thus playing a role in the pathophysiology of sepsis and asthma.

The NOP receptor is found in abundant quantities in the forebrain, the midbrain, the dorsal and ventral horns of the spinal cord and the brain stem. These are largely observed in pain-related brain regions, both ascending and descending pathways and they are also present in significant amounts in regions that mediate reward.

In addition to pain, reward, stress and anxiety, the NOP receptor is involved in many other central processes such as learning and memory, emotional states, neuroendocrine regulation, food intake, and motor control.

The effect of N/OFQ is mainly influenced by two factors: the dose and the route of administration. If N/OFQ is administered i.c.v. to rodents, it induces hyperalgesia and allodynia, and blocks the analgesic effect of MOP, DOP and KOP receptors. In the spinal cord N/OFQ has bidirectional effects, since it shows a pronociceptive effect in very low (femtomolar) concentrations, while in higher (nanomolar) dosage it has an analgesic effect. It is postulated that achievement of an antinociceptive effect through inhibition of the NOP receptor is only possible using i.c.v. administered NOP receptor antagonists.

Since the discovery of morphine, a number of new opioid receptor drugs have been developed with the aim of eliminating the severe and unpleasant side effects of opioid pharmaceutics. Of the opioid receptors, the MOP receptor plays the most important role and new drugs have been developed for this receptor. These new ligands have more or less shown the side effects characteristic to morphine in human medicine. Some of these pharmaceuticals and their side effects are summarized in **Table 1**.

Table 1: MOP receptor drugs and their common side effects.

MOPr drugs	**Common side effects**
Codeine	lightheadedness, dizziness, sedation, shortness of breath, nausea, vomiting, sweating, constipation
Fentanyl	fever, respiratory depression, nausea, vomiting, diaphoresis
Levorphanol	nausea, vomiting, altered mood, pruritus, flushing, difficulties in urination, constipation, biliary spasm
Meperidine	lightheadedness, dizziness, sedation, nausea, vomiting, sweating

Methadone	euphoria, sedation, respiratory depression, miosis, bradycardia, physical dependence
Morphine	drowsiness, dizziness, sedation, fever, anxiety, confusion, tremor, diaphoresis, lethargy, feeling of warmth, respiratory depression, dry mouth, constipation, nausea, diarrhea, anorexia, abdominal pain, vomiting, chest pain, anemia, leukopenia, rash, peripheral eodema
Oxycodone	constipation, nausea, somnolence, dizziness, vomiting, pruritus, headache, dry mouth, asthenia, sweating, nausea, headache, pyrexia constipation
Oxymorphone	nausea, pyrexia, somnolence, vomiting, pruritus, headache, dizziness, constipation, confusion, diarrhea, insomnia, fatigue, decreased appetite, abdominal pain
Tramadol	nausea, constipation, dry mouth, somnolence, dizziness, vomiting

Besides ligands acting on one of the receptors, mixed/bifunctional and bivalent opioid ligands were also developed. These ligands target more than one opioid receptors, which hopefully enables them to be more effective and helps reduce their side effects. Mixed opioid ligands currently on the market show somewhat milder adverse effects, though they are still frequently involved in drug abuse (**Table 2**).

Table 2: Mixed Opioid drugs and their side effects.

Mixed opioid agonist/antagonist drugs	**Receptor effect**	**Common side effects**
Buprenorphine	partial agonist at MOPr and NOPr, antagonist at KOPr and DOP	headache, insomnia, pain, withdrawal, nausea, constipation, application site pruritus, vomiting, hyperhidrosis, dizziness, somnolence, dry mouth, rash, abuse
Butorphanol	antagonist at MOPr, agonist at KOPr	somnolence, dizziness, nausea, vomiting, abuse
Nalbuphine	antagonist at MOPr agonist at KOPr	sweaty, clammy, nausea, vomiting, dizziness, vertigo, dry mouth, headache, abuse
Pentazocine	antagonist at MOPr agonist at KOP	nausea, dizziness,lightheadedness, vomiting, euphoria, abuse

Potential strategies include targeting receptor dimers with bifunctional or bivalent ligands. Bifunctional and bivalent drugs are not clearly distinguished from each other in the literature, and are sometimes used as synonyms. According to Dietis et al., bifunctional ligands are designed to be non-selective compounds that comprise two protein-binding drug moieties (with or without a spacer) in one chemical structure and act at two different therapeutic targets.

Bivalent ligands, on the other hand, are intended to be selective compounds, which composed of two distinct pharmacophores joined by a linker and usually have a larger molecular weight than bifunctional ligands. They are usually designed to bind to their two targets, which is not always achieved in practice. In the present thesis, the term *bifunctional* shall be used to refer to our newly synthesized ligands.

One of the most preferred targets among opioid receptors is the MOP receptor. MOP/DOP, MOP/NOP, MOP/CCK2, MOP/NK1 and MOP/NTS receptor bivalent ligands have been developed to reduce side effects while maintaining the MOP receptor-mediated analgesic effects.

In addition to these bivalents, MOP/NOP bivalent ligands are also widely researched. NOP receptor agonist and partial agonist ligands are both used for constructing bivalents of the MOP/NOP receptor.

Among the NOP/MOP receptor agonists, the bifunctional ligand cebranopadol has been found to be effective even in the nanomolar range on the MOP, DOP, KOP and NOP receptors. In *in vivo* tests, it has proved to be an effective substance. This compound is currently in phase II and III clinical trials investigating the efficacy, safety and tolerability of the orally administered drug.

Ac-RYYRIK-NH_2 was in the focus of interest several times when MOP/NOP and other OP/NOP receptor bivalent ligands were developed. These bivalent ligands were designed to study the heterodimerization of opioid and NOP receptors, namely, the organization of the heterodimers; to develop new therapeutic agents, and to reduce morphine-related side effects.

Kawano et al. used dermorphine besides Ac-RYYRIK-NH_2 to form a MOP receptor agonist and NOP receptor antagonist ligand. Analogue 1, which they have created, binds to the MOP receptor with 200 times higher affinity than the starting ligands, and to the NOP receptor with 17 times higher affinity.

Guillemyn et al. developed an effective MOP agonist and weak NOP antagonist peptide ligand 13a (H-Dmt-D-Arg-Aba-β-Ala-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2). Intravenous 13a has a more prolonged effect than morphine. It had superior anti-allodynic and anti-hyperalgesic properties intrathecally than either morphine or Ac-RYYRIK-NH_2. Overall, this ligand can already be effective at nanomolar concentrations and may be useful for both acute and neuropathic pain.

Similarly to the previous research groups, Lagard et al. also used Ac-RYYRIK-NH_2 to form their MOP/NOP receptor bivalent ligands. After acute and neuropathic pain tests, KGNOP1 (H-Dmt-D-Arg-Aba-bAla-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2) proved to be the most

promising as its analgesic effect was more effective than that of even tramadol or morphine. In addition, its effect on respiratory depression was minimal.

AIMS OF THE STUDIES

The N/OFQ-NOP receptor system is widely distributed in both the central and peripheral nervous system, the airways, the cardiovascular system, the urogenital and gastrointestinal tract, and the immune system. Since the N/OFQ-NOP receptor system is implicated in the regulation of numerous different biological functions in different ways, both NOP receptor agonists, antagonists and partial agonists are potentially useful tools for treating NOP receptor-related disorders.

In the present thesis 12 newly synthesized peptide ligands are described, 9 of which are N/OFQ variants and 3 of which OP/NOP receptor bifunctional ligands. 4 of the N/OFQ variants only contained elements of the N/OFQ sequence, while the remaining 5 also contained parts from the Ac-RYYRIK-NH_2 sequence.

<u>The objectives of this study were set out as follows:</u>

- to characterize the **binding affinity** of 3 bifunctional ligands towards MOP, DOP, KOP and NOP receptors and the 9 fused N/OFQ hybrid peptides towards NOP receptor on rat and guinea pig brain membranes,

- and to investigate the opioid receptors mediated **G-protein activity** in a functional [^{35}S]GTPγS binding assay, and to investigate the **pharmacological activity** on isolated MVD (mouse *vas deferens*), which contains all opioid receptors, including NOP receptor.

METHODS

Competition radioligand binding experiments

In competition binding experiments, the affinity (K_i) of an unlabeled compound is analysed by measuring radioligand specific binding in the presence of increasing concentrations of the unlabelled compound in question. The experimental data were analyzed and points were fitted with the professional curve fitting program GraphPad Prism 6.0 using non-linear regression.

The tested ligands and their parent compounds (YGGF, YGGFL, nociceptin, Ac-RYYRIK-NH_2) were examined in rat and guinea pig brain membrane homogenates using highly MOP, DOP, KOP and NOP receptor selective ligands.

The competition binding assays were performed in duplicates and repeated at least three times.

[^{35}S]GTPγS binding experiments

The [^{35}S]GTPγS binding assay allows studying G protein activation. In this assays GDP→GTP exchange is measured using non-hydrolysable, radioactive [^{35}S]GTPγS in the presence of the tested ligand in a given concentration. The measured radioactive signal corresponds to basal activity (100%) in the absence of an agonist. The [^{35}S]GTPγS binding experiments also provided opportunity to study antagonism by MOP, DOP, KOP and NOP receptor selective ligands. The G-protein activity of opioid-nociceptin and nociceptin hybrid peptides were measured in rat and guinea pig brain membrane fractions.

The maximal stimulation (E_{max}) of the G-proteins and the potency (EC_{50}) of the tested ligands were calculated from the specifically bound [^{35}S]GTPγS in the presence of the increasing concentrations of tested ligands.

Isolated mouse *vas deferens (MVD)* bioassay

This mouse *vas deferens* bioassay is used to assess the parameters of the ligands such as agonist potency and selectivity, which allow us to distinguish between full agonists, partial agonists and antagonists.

To determine the equilibrium dissociation constant (K_e) of antagonists, the ‘single-dose’ method was applied.

SUMMARY OF THE RESULTS

Bifunctional ligands

- We concluded that the three bifunctional ligands bound to the DOP, KOP, NOP receptors even in a small concentration. **BA55** had the highest affinity to the KOP and NOP receptors, **BA61** to the NOP and DOP receptors, and **BA62** to the DOP and KOP receptors.

- The G-protein activation tests showed that both **BA55** and **BA62** stimulated the G-proteins effectively, which was mediated by the DOP, KOP and NOP receptors. The stimulation of G-proteins by **BA61** was weaker, and could be mediated through the DOP and NOP receptors, and, to a lesser extent, the MOP receptor.

- Based on the results of the MVD bioassays, we deduced that all three ligands preferred the DOP and KOP receptors to the MOP and NOP receptors. According to the K_e values, **BA61** showed agonist activity on the DOP and NOP receptor, while **BA62** was the strongest agonist of the KOP receptor.

Nociceptin analogues and nociceptin-RYYRIK hybrid peptides

- The radioligand competition binding assays showed that the ligands that showed greater NOP receptor affinity than the parent compounds were **P1, P2, P4, P6** and **P7**.

- In the G-protein activation tests, the **P2** and **P3** peptides showed full agonist effect on the NOP receptor. The remaining 7 peptides behaved as partial agonists.

- In the MVD bioassay **P1, P4, P5**, **P6** and **P7** behaved as partial agonists. **P4, P5** and **P6** inhibited the effect of N/OFQ to a greater extent than the control JTC-801.

CONCLUSION

Bivalent ligands acting on opioid receptors are an important area of research, since mixed or bifunctional compounds similar to bivalent ligands have already been used successfully in human medicine. In addition, compounds acting on the NOP receptor play a crucial role in the investigation of the N/OFQ-NOP system and its physiological effects, such as tolerance and dependence.

We have synthesized multiple novel bifunctional ligand candidates, among which **BA61** proved to be promising as it was able to bind to the DOP receptor and weakly to the NOP receptor, even though the opioid message domain was at an unusual location.

Among the 9 N/OFQ variants, **P1, P4, P5, P6**, and **P7** showed the highest affinity towards the NOP receptor. In addition, **P4, P5, P6**, and **P7** were efficient NOP receptor antagonists in the mouse *vas deferens* bioassay. These ligands carry the 'Ac-RYYRIK' motif at the N-terminus, which suggests that 'Ac-RYYRIK' can work in place of the nociceptin message domain.

Further *in vitro* and *in vivo* studies, such as rat tail-flick tests and gastrointestinal motility tests are needed to understand the exact pharmacology and possible clinical utility of the above mentioned peptides.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **Erdei AI**, Borbely A, Magyar A, Taricska N, Perczel A, Zsiros O, Garab G, Szucs E, Otvos F, Zador F, Balogh M, Al-Khrasani M, Benyhe S. Biochemical and pharmacological characterization of three opioid-nociceptin hybrid peptide ligands reveals substantially differing modes of their actions. PEPTIDES 99: pp. 205-216. (2018)

II. **Erdei AI**, Borbely A, Magyar A, Szűcs E, Ötvös F, Gombos D, Al-Khrasani M, Stefanucci A,Dimmito M.P, Luisi G, Mollica A, Benyhe S. Biochemical and pharmacological investigation of novel nociceptin/OFQ analogues and N/OFQ-RYYRIK hybrid peptides. PEPTIDES (2018).

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