Closing report on

OTKA-108518 NKFI research project entitled

„Cellular and molecular interactions of bivalent ligands with oligomer receptors”

Multitarget ligands, also called bivalent or hybrid compounds represent a novel class of bioactive molecules. They are composed of various pharmacophores and are capable of interacting with different target proteins.

The objective of the research project was to provide a detailed molecular and functional description of novel peptide-based communication pathways that are thought to modulate a number of physiological and pathological processes including pain, mood, and autonomic functions in the central nervous system. Novel peptide ligands for the opioid and nociceptin receptors, including structural analogues, selective antagonists, radioprobes were developed and studied by means of synthetic-chemical and functional-biochemical methods. Numerous studies have established the presence of G-protein coupled receptors (GPCRs) as dimers in heterologous cell expression systems but also in vivo. In this context, heterodimers of opioid receptors would constitute new targets of specific interest, each entity possessing original properties in terms of function and pharmacology. Mu opioid receptor (MOPr) activation induces analgesia, while NOPr activation would produce hyperalgesia. Thus, in order to fight pain, it would be important to synthesize one molecule combining the agonist effect for the MOP and antagonist for the NOP receptors, in order to reinforce the synergistic effect on analgesia. The objective of this project was to conceive new analgesic molecules by combining two pharmacophores targeting the MOPr/NOPr and other heterodimers. These bivalent ligands would be part of a new generation of drugs, at least as efficient as morphine and maybe deprived of some side effects.

A number of novel multitarget ligands has been designed, developed and studied during the project. This research has been initiated and cunducted in Hungary, but the research-development programme involved also international participants mainly from Poland and Italy.

The following main achievements in developing multitarget compounds have been made:

i/ Characterization of three hybrid peptides interacting with NOP and MOP receptors.

In an attempt to design opioid-nociceptin hybrid peptides, three novel bivalent ligands, H-YGGFGGGRYYRIK-NH_2, H-YGGFRYYRIK-NH_2 and Ac-RYYRIKGGGYGGFL-OH were synthesized and studied by biochemical, pharmacological, biophysical and molecular modelling tools. These chimeric molecules consist of YGGF sequence, a crucial motif in the N-terminus of natural opioid peptides, and Ac-RYYRIK-NH_2, which was isolated from a combinatorial peptide library as an antagonist or partial agonist that inhibits the biological activity of the endogenously occurring heptadecapeptide nociceptin. Solution structures for the peptides were studied by analysing their circular dichroism spectra. Receptor binding affinities were measured by equilibrium competition experiments using four highly selective radioligands. G-protein activating properties of the multitarget peptides were estimated in [^{35}S]GTPγS binding tests. The three compounds were also measured in electrically stimulated mouse vas deferens (MVD) bioassay. H-YGGFGGGRYYRIK-NH_2 (BA55), carrying N-terminal opioid and C-terminal nociceptin-like sequences interconnected with GGG tripeptide spacer displayed a tendency of having either unordered or β-sheet structures, was moderately potent in MVD and possessed a NOP/KOP receptor preference. A similar peptide without spacer H-YGGFRYYRIK-NH_2 (BA62) exhibited the weakest effect in MVD, more α-helical periodicity was present in its structure and it exhibited the most efficacious agonist actions in the G-protein stimulation assays. The third hybrid peptide Ac-RYYRIKGGGYGGFL-OH (BA61) unexpectedly displayed opioid receptor affinities, because the opioid message motif is hidden within the C-terminus. The designed chimeric peptide ligands presented in this study accommodate well into a group of multitarget opioid compounds that include opioid-non-opioid peptide dimer analogues, dual non-peptide dimers and mixed peptide- non-peptide bifunctional ligands. (Erdei et al., 2017)

ii/ Cyclic biphalin analogues

In this work we enhanced the ring lipophilicity of biphalin introducing a xylene moiety, thus obtaining three cyclic regioisomers. Novel compounds have similar in vitro activity as the parent compound, but one of these (6a) shows a remarkable increase of in vivo antinociceptive effect. Nociception tests have disclosed its significant high potency and the more prolonged effect in eliciting analgesia, higher than that of biphalin and of the disulfide-bridge-containing analogue (7). (Stefanucci et al., 2017) Italian cooperation.

iii/ Exploring the first Rimonabant analog-opioid peptide hybrid compound, as bivalent ligand for CB1 and opioid receptors

Cannabinoid (CB) and opioid systems are both involved in analgesia, food intake, mood and behavior. Due to the co-localization of μ-opioid (MOR) and CB1 receptors in various regions of the central nervous system (CNS) and their ability to form heterodimers, bivalent ligands targeting to both these systems may be good candidates to investigate the existence of possible cross-talking or synergistic effects, also at sub-effective doses. In this work, we selected from a small series of new Rimonabant analogs one CB1R reverse agonist to be conjugated to the opioid fragment Tyr-D-Ala-Gly-Phe-NH_2. The bivalent compound (9) has been used for in vitro binding assays, for in vivo antinociception models and in vitro hypothalamic perfusion test, to evaluate the neurotransmitters release. (Mollica et al., 2017) Italian cooperation.

iv/ Antinociceptive effect induced by a combination of opioid and neurotensin moieties vs. their hybrid peptide [Ile(9)]PK20 in an acute pain treatment in rodents

Hybrid compounds are suggested to be a more effective remedy for treatment of various diseases than combination therapy, since the attenuation or total disappearance of side effects, typically induced by a single moiety, can be observed. This is of great importance, especially when we consider problems resulting from the use of opioid analgesics. However, although it seems that such compounds can be valuable therapeutic tools, the lack of conviction among the public as to the appropriateness of their use still remains; therefore patients are commonly treated with polypharmacy. Thus, in the presented paper we show a comparison of the antinociceptive effect between a novel opioid-neurotensin chimera called [Ile(9)]PK20 and a mixture of its structural elements, delivered intrathecally and systemically. Additionally, motor coordination was assessed in the rotarod test. The results clearly indicate that spinal administration of the examined compounds, resulted in a long-lasting, dose- and time-dependent antinociceptive effect. Although the mixture of both pharmacophores was found to be more active than [Ile(9)]PK20, motor impairments surfaced as a side effect. This in turn illustrates the advantageous use of hybrid structures over drug cocktails. (Kleczkowska et al., 2016) Polish cooperation.

v/ Biological evaluation and molecular docking studies of AA3052, a compound containing a μ-selective opioid peptide agonist DALDA and d-Phe-Phe-d-Phe-Leu-Leu-NH2, a substance P analogue

The design of novel drugs for pain relief with improved analgesic properties and diminished side effect induction profile still remains a challenging pursuit. Tolerance is one of the most burdensome phenomena that may hamper ongoing opioid therapy, especially in chronic pain patients. Therefore, a promising strategy of hybridizing two pharmacophores that target distinct binding sites involved in pain modulation and transmission was established. Previous studies have led to the development of opioid agonist/NK1 agonist hybrids that produce sufficient analgesia and also suppress opioid-induced tolerance development. In our present investigation we assessed the antinociceptive potency of a new AA3052 chimera comprised of a potent MOR selective dermorphin derivative (DALDA) and an NK1 agonist, a stabilized substance P analogue. We have shown that AA3052 significantly prolonged responses to both mechanical and noxious thermal stimuli in rats after intracerebroventricular administration. Additionally, AA3052 did not trigger the development of tolerance in a 6-day daily injection paradigm nor did it produce any sedative effects, as assessed in the rotarod performance test. However, the antinociceptive effect of AA3052 was independent of opioid receptor stimulation by the DALDA pharmacophore as shown in the agonist-stimulated G-protein assay. Altogether the current results confirm the antinociceptive effectiveness of a novel opioid/SP hybrid agonist, AA3052, and more importantly its ability to inhibit the development of tolerance. (Kowalczyk et al., 2016) Polish cooperation.

vi/ Evaluation of the analgesic effect of 4-anilidopiperidine scaffold containing ureas and carbamates

Fentanyl is a powerful opiate analgesic typically used for the treatment of severe and chronic pain, but its prescription is strongly limited by the well-documented side-effects. Different approaches have been applied to develop strong analgesic drugs with reduced pharmacologic side-effects. One of the most promising is the design of multitarget drugs. In this paper we report the synthesis, characterization and biological evaluation of twelve new 4-anilidopiperidine (fentanyl analogues). In vivo hot-Plate test, shows a moderate antinociceptive activity for compounds OMDM585 and OMDM586, despite the weak binding affinity on both μ and δ-opioid receptors. A strong inverse agonist activity in the GTP-binding assay was revealed suggesting the involvement of alternative systems in the brain. Fatty acid amide hydrolase inhibition was evaluated, together with binding assays of cannabinoid receptors. We can conclude that compounds OMDM585 and 586 are capable to elicit antinociception due to their multitarget activity on different systems involved in pain modulation. (Monti et al., 2016) Italian cooperation.

vii/ Design, Synthesis and Biological Evaluation of Two Opioid Agonist and Cav 2.2 Blocker Multitarget Ligands

N-type voltage-dependent Ca(2+) channels (CaV 2.2) are located at nerve endings in the central and peripheral nervous systems and are strongly associated with the pathological processes of cerebral ischaemia and neuropathic pain. CaV 2.2 blockers such as the ω-conotoxin MVIIA (Prialt) are analgesic and have opioid-sparing effects. With the aim to develop new multitarget analgesic compounds, we designed the first ω-conotoxin/opioid peptidomimetics based on the enkephalin-like sequence Tyr-D-Ala-Gly-Phe (for the opioid portion) and two fragments derived from the loop-2 pharmacophore of ω-conotoxin MVIIA. Antinociceptive activity evaluated in vitro and in vivo revealed differential affinity for CaV 2.2 and opioid receptors and no significant synergistic activity. (Mollica et al., 2015) Italian cooperation.

viii/ Novel cyclic biphalin analogue with improved antinociceptive properties

Two novel opioid analogues have been designed by substituting the native d-Ala residues in position 2,2' of biphalin with two residues of d-penicillamine or l-penicillamine and by forming a disulfide bond between the thiol groups. The so-obtained compound 9 containing d-penicillamines showed excellent μ/δ mixed receptor affinities (K i (δ) = 5.2 nM; K i (μ) = 1.9 nM), together with an efficacious capacity to trigger the second messenger and a very good in vivo antinociceptive activity, whereas product 10 was scarcely active. An explanation of the two different pharmacological behaviors of products 9 and 10 was found by studying their conformational properties. (Mollica et al., 2014) Italian cooperation.

Besides multitarget ligands, a number of more traditional, although specific bioactive compounds have been developed and studied. These compounds would be pharmacophores of further bivalent molecules in the near future. These inactive and radiolabelled compounds include hemopressin peptide fragments (Szlávicz et al., 2015), [^{3}H]neuromedin (Tóth et al., 2016), naltrexone-O-14-sulphate (Zádor et al., 2017a), 14-O-methyl-morphine (Zádor et al., 2017).

Further research on opioid-based bivalent compounds will include various kynurenine analogues, targeting the neuronal NMDA receptors. This research is partly supported also by NKFI (GINOP). Preliminary research on the interaction between the opioid system and kynurenines have been accomplished during the project and resulted in some publications:

Kynurenic acid and its analogue can alter the opioid receptor G-protein signaling after acute treatment via NMDA receptor in rat cortex and striatum (Samavati et al., 2017); Interactions between the Kynurenine and the Endocannabinoid System with Special Emphasis on Migraine (Nagy-Grócz et al., 2017).

Finally, a pioneer study on the interaction of the cannabinoid and the opioid system is mentioned as a result of the recently finished NKFI project: Low dosage of rimonabant leads to anxiolytic-like behavior via inhibiting expression levels and G-protein activity of kappa opioid receptors in a cannabinoid receptor independent manner. (Zádor et al., 2015)