TARGETING THE CANNABINOID AND MU OPIOID RECEPTORS WITH HETERODIMERIZED AND ALLOSTERIC LIGANDS

Summary of the Ph.D. Thesis

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INTRODUCTION

Mu opioid receptor (MOR) agonists are the most common therapeutics in clinic to alleviate severe pain. However, their dose-limiting adverse effects inspire the development of novel analgesics. Cannabinoid (CB) receptor agonists can modulate hyperalgesia and show effective therapeutic value against inflammatory and chronic pain including neuropathic pain. The co-administration of MOR and CB receptor agonists has been shown to enhance the antinociceptive effect with decreased opiate-related side-effects, and the synergism of opioid and cannabinoid ligands has been extensively studied in mice, in rats, in rhesus monkeys and in an experimental pain model applied to volunteers.

Initiated by the possible receptor dimerization interaction of the opioid and cannabinoid receptors bivalent compounds, i.e. spacer linked pharmacophores, were considered to decrease the opioid side-effects. Conjugating the MOR agonist fentanyl to rimonabant, a CB1 antagonist/inverse agonist resulted in MOR–CB antagonists. Coupling of an enkephalin-related peptide to rimonabant led to the loss of analgesic effects in hot plate and tail flick tests. In contrast, bivalent compounds of the MOR agonist α-oxymorphamine and a rimonabant analogue were found to exhibit antinociception in tail flick test without producing tolerance in 24 h.

Another important goal of the combination treatments is to decrease the effective dose of opioids, especially in the treatment of severe chronic pains. It could be potentially achieved by the combination of opioid agonists with cannabinoid agonists.

In order to target the MOR and the CB receptors with a single compound, bivalent ligands consisting of a MOR and a CB agonist were designed. In one set the MOR agonist oxycodone, that is widely used in the treatment of severe pain was applied. The other set contained the enkephalin-related tetrapeptide Tyr-D-Ala-Gly-Phe as the opioid pharmacophore. Both opioid agonists were combined with naphthalen-1-yl(1-pentyl-1H-indol-3-yl)methanone (JWH-018), a full CB agonist. This indole-type cannabimimetic binds to both the CB1 and CB2 receptors with low nanomolar affinity, and exhibits in vivo cannabinoid pharmacological effects.

The recently discovered α-hemoglobin derived hemopressins have been postulated to be negative allosteric modulators and endogenous agonist ligands of the CB1 receptors. These peptides have been demonstrated to possess in vitro and in vivo pharmacological potencies similar to those of the prototypic endogenous and synthetic cannabinoid ligands, but with less side-effects. Accordingly, hemopressins appear to be excellent lead

compounds for the development of peptidic research tools for the investigation of the endocannabinoid system.

Due to these favorable characteristics and to the fact that the truncated Hp(1–7) peptide was also found to be as potent as Hp(1–9) in in vitro and in vivo studies, Hp(1–7) was chosen for radiolabelling. The resulting novel radioligand was investigated in various radioligand binding assays to characterize the interaction of Hp(1-7) and CB receptors.

AIMS

Mu opioid receptor (MOR) agonists are the most common therapeutics clinically used to alleviate pain. However, their dose-limiting adverse effects including respiratory depression, sedation, constipation, tolerance and dependence inspires the development of novel analgesics. Combination therapy has been demonstrated to be effective for improving analgesic effects without the additive elevation of the side-effects. The co-administration of MOR and CB receptor agonists has been shown to result in enhanced antinociceptive effect with decreased opiate-related side-effects, and the synergism of opioid and cannabinoid ligands has been extensively studied to improve antinociception.

<u>The aims of the study presented here were the following:</u>

- To design and synthesize two series of bivalent ligands for targeting both the MOR and the CB receptors.
- To label the cannabinoid pharmacophore (JWH-018) of the bivalent ligands and the truncated hemopressin heptapeptide (Hp1-7) with tritium for direct *in vitro* characterization of their receptor binding on rat and mouse brain membrane homogenates.

- To compare the binding sites of classical CB ligands and hemopressins in displacement assay using [^{3}H]JWH-018 and [^{3}H]Hp(1-7).
- To study the effects of the modifications of the synthetic bivalent ligands and hemopressins on the receptor affinity and selectivity in binding studies.
- To study the agonist/antagonist properties and the MOR, CB1/CB2 mediated signaling of the bivalent compounds and hemopressins using ligand-stimulated [^{35}S]GTPγS functional assay.
- To investigate the permeability of selected bivalent derivatives through the blood brain barrier.
- To test the *in vivo* antinociceptive effects of the *in vitro* most effective bivalent ligands.

MATERIALS AND METHODS

Preparation of monomeric and bivalent compounds

The oxycodone – JWH-018 (**10**-**12**) and the peptide – JWH-018 (**18**-**21**) bivalent compounds were prepared in a convergent way, and the conjugation of the opioid and cannabinoid pharmacophore units was performed via spacers of different length (2-13 atoms) and polarity. Details of the preparation and analytical characterization of compounds **1**-**25** are described in the appendix of the Ph.D. Thesis.

Preparation of hemopressins on solid support

The solid phase peptide syntheses of Hp(1–7), ΔPro^{1}-Hp(1–7), (Hp(1–9) and RVD-Hp(1–9) were carried out manually by Boc/Bzl or Fmoc/*t*-Bu chemistry.

Radiolabeling of JWH-018 and hemopressin(1–7)

JWH-018 and the precursor peptide ΔPro^{1}-Hp(1–7) was labeled with tritium under heterogenous conditions.

Characterization of the novel CB receptor radioligands [^{3}H]JWH-018 and [^{3}H]hemopressin(1-7)

Before their application in radioligand competition assays, [^{3}H]JWH-018 and [^{3}H]hemopressin(1-7) were characterized in various *in vitro* receptor binding experiments.

Association and dissociation binding experiments were performed

to characterize the interaction of [^{3}H]JWH-018 and [^{3}H]hemopressin(1-7) with membrane receptors using rat brain membrane homogenates. Saturation binding experiments were then performed on brain homogenates of rat and CB1 knockout mouse to determine the K_d and B_{max} values.

Radioligand binding assays

In order to assess the effects of the structural changes of the monomeric ligands on the biological activity, and to evaluate the bivalent compounds for receptor affinity and selectivity, the novel synthetic compounds were subjected to radioligand binding assays.

Radioligand competition binding assays

Displacements of the MOR selective radioligand [^{3}H]DAMGO, the DOR selective [^{3}H]Ile^{5,6}-deltorphin-2, the KOR selective [^{3}H]HS-665 and the CB receptor radioligands [^{3}H]JWH-018 and [^{3}H]WIN-55,212-2 by the synthetic compounds were investigated in rat or guinea pig brain membrane homogenates.

Next, competition experiments were performed to investigate the ability of classical CB receptor ligands and hemopressins Hp(1-7), Hp(1-9) and RVD-Hp(1-9) to inhibit the binding of [^{3}H]Hp(1-7) to rat and CB1 knockout mouse brain membrane homogenate.

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

The signaling properties of the bivalent compounds and hemopressins were investigated in ligand-stimulated [^{35}S]GTPγS

binding experiments in rat and CB1 knockout mouse brain membrane homogenate.

To explore the activation of MOR and/or CB1/CB2 receptor-mediated signaling induced by **11** and **19**, the G-protein activation was investigated in the absence or presence of antagonists (10 μM naloxone, 10 μM rimonabant or 10 μM AM 630) in rat brain membrane homogenate.

SUMMARY AND CONLUSION

- ✓ Two series of bivalent compounds containing an opioid (oxycodone or Tyr-D-Ala-Gly-Phe) and a cannabinoid (JWH-018) pharmacophore were designed, synthesized and characterized in *in vitro* radioligand binding assays, functional [^{35}S]GTPγS binding assays and *in vivo* antinociceptive tests.
- ✓ Two novel CB receptor radioligands, [^{3}H]JWH-018 and [^{3}H]Hp(1-7) were prepared and validated.
- ✓ It was found that [^{3}H]JWH-018 bound to the CB receptor binding site with high affinity (K_d= 6.5 nM) and fast kinetics and it labeled high receptor density (B_{max}= 1120 ± 89 fmol/mg protein). In displacement studies [^{3}H]JWH-018 competed with the classical orthosteric CB receptor ligands but not with hemopressins and opioid ligands.
- ✓ [^{3}H]Hp(1-7) displayed saturable binding in rat brain membrane and also in a CB1 knockout mouse brain homogenate. The receptor bound [^{3}H]Hp(1-7) could not be displaced by JWH-018, rimonabant and AM251.
- ✓ The *C*-6 substitution of oxycodone did not significantly affected the MOR binding and MOR selectivity, but led to loss of KOR affinity. The introduction of spacers with increasing length and polarity slightly reduced the MOR affinity and selectivity.

- ✓ The introduction of a terminal carboxyl, amino and acylamido function to the pentyl chain of JWH-018 resulted in 73-fold, 55-fold and 43-fold loss of CB receptor affinity, respectively.
- ✓ The functional binding assays revealed that the *C*-6 substitution of oxycodone and the conjugation of linkers to this position reduced the G-protein activation efficacy and led to weak partial agonists with lower potency.
- ✓ The modification of the full agonist JWH-018 with a carboxyl, an amino or acylamido group resulted in inverse agonist or antagonist ligands.
- ✓ In competition binding assays the affinity and selectivity of the bivalent compounds **10** and **12** to the MOR slightly decreased and their CB receptor affinity was even lower.
- ✓ The MOR affinity and selectivity of the bivalent compounds **18**, **20**, **21** decreased and so did their CB receptor affinity.
- ✓ In the functional binding assays the bivalent compounds **10** and **12** were found to be antagonists, whereas **18**, **20**, **21** acted as partial agonists.
- ✓ In competition binding assays the bivalent compounds **11** (K_i (MOR)= 18 nM; K_i (CB)= 34 nM) and **19** (K_i (MOR)= 2.1 nM; K_i (CB) = 251 nM) showed the highest affinity both to the MOR and to the CB receptors.

- ✓ In functional binding assays it was found that the agonist bivalent compound **11** exerted its G-protein activation through the MOR and CB2 receptors, while the agonist bivalent compound **19** exerted its G-protein activation through the MOR and CB1 receptors.
- ✓ Dimerization of MOR and CB agonists resulted in the agonist bivalent compounds **11** and **19** with antiallodynic activity *in vivo* during the early and late phase.
- ✓ At spinal level bivalent compound **11** and **19** were equieffective with the parent drugs at 20 μg dose in a chronic osteoarthritis pain model in rats.
- ✓ Because MOR and CB receptor agonists can be effectively applied in the treatment of chronic pain including neuropatic pain, these findings can help to develop multitargeting antinociceptive drugs.
- ✓ In the future, the hemopressin peptide family can be applied in bivalent molecules as a CB receptor targeting moiety probably without cannabinoid side effects.

LIST OF PUBLICATIONS

List of thesis related publications:

I. Dvorácskó, Sz., Keresztes, A., Mollica, A., Stefanucci, A., Macedonio, G., Pieretti, S., Zádor, F., Walter, F., Deli, M., Kékesi, G., Bánki, L., Tuboly, G., Horváth, Gy., Tömböly, Cs. **Preparation of bivalent agonists for targeting the mu opioid and cannabinoid receptors** *Eur. J. Med. Chem.* submitted (2019).

II. Dvorácskó, Sz., Tömböly, Cs., Berkecz, R., Keresztes, A. **Investigation of the receptor binding and functional characteristics of hemopressin(1-7)**. *Neuropeptides* **58**, 15-22 (2016). **IF: 2,915**

III. Dvorácskó, Sz., Stefanucci, A., Novellino, E., Mollica, A. **Design of multi target ligands for chronic and neuropathic pain**. *Future Med. Chem.* **7**, 2469-83 (2015). **IF: 3,969**

Other publications:

1. Mollica, A., Mirzaie, S., Costante, R., Carradori, S., Macedonio, G., Stefanucci, A., Dvorácskó, Sz., Novellino, E. Exploring the biological consequences of conformational changes in aspartame models containing constrained analogues of phenylalanine. *J. Enzyme Inhib. Med. Chem.* **31**, 953-63 (2016). **IF: 3,638**

2. Monti, L., Stefanucci, A., Pieretti, S., Marzoli, F., Fidanza, L., Mollica, A., Mirzaie, S., Carradori, S., De Petrocellis, L., Schiano, M. A., Benyhe, S., Zádor, F., Szűcs, E., Ötvös, F., Erdei, A., Samavati, R., Dvorácskó, Sz., Tömböly, Cs., Novellino, E. Evaluation of the analgesic effect of 4-anilidopiperidine scaffold containing ureas and carbamates. *J. Enzyme Inhib. Med. Chem*. **31**, 1638-47 (2016). **IF: 3,638**

3. Stefanucci, A., Costante, R., Macedonio, G., Dvorácskó, Sz., Mollica, A. Cysteine-, methionine- and seleno-cysteine-proline chimeras: Synthesis and their use in peptidomimetics design. *Curr. Bioact. Comp.* **12**, 200-206 (2016). **IF: 0**

4. Szűcs, E., Dvorácskó, Sz., Tömböly, Cs., Büki, A., Kékesi, G., Horváth, Gy., Benyhe, S. Decreased CB receptor binding and cannabinoid signaling in three brain regions of a rat model of schizophrenia. *Neurosci. Lett*. **633**, 87-93 (2016). **IF: 2,159**

5. Mollica, A., Pelliccia, S., Famiglini, V., Stefanucci, A., Macedonio, G., Chiavaroli, A., Orlando, G., Brunetti, L., Ferrante, C., Pieretti, S., Novellino, E., Benyhe, S., Zádor, F., Erdei, A., Szűcs, E., Samavati, R., Dvorácskó, Sz., Tömböly, Cs., Ragno, R., Patsilinakos, A., Silvestri, R. Exploring the first Rimonabant analog-opioid peptide hybrid compound, as bivalent ligand for CB1 and opioid receptors. *J. Enzyme Inhib. Med. Chem*. **32**, 444–451 (2017). **IF: 3,638**

6. Nagy-Grócz, G., Zádor, F., Dvorácskó, Sz., Bohár, Zs., Benyhe, S., Tömböly, Cs., Párdutz, Á., Vécsei, L. Interactions between the Kynurenine and the Endocannabinoid System with Special Emphasis on Migraine. *Int. J. Mol. Sci.* **18**, e1617 (2017). **IF: 3,687**

7. Adamska-Bartłomiejczyk, A., Borics, A., Tömböly, Cs., Dvorácskó, Sz., Lisowski, M., Kluczyk, A., Wołczański, G., Piekielna-Ciesielska, J., Janecka, A. Synthesis, receptor binding studies, optical spectroscopic and in silico structural characterization of morphiceptin analogs with cis-4-amino-L-proline residues. *J. Pept. Sci.* **23**, 864-870 (2017). **IF: 1,969**

8. Leone, S., Recinella, L., Chiavaroli, A., Martinotti, S., Ferrante, C., Mollica, A., Macedonio, G., Stefanucci, A., Dvorácskó, Sz.,

Tömböly, Cs., De Petrocellis, L., Vacca, M., Brunetti, L., Orlando, G. Emotional disorders induced by Hemopressin and RVD-hemopressin(α) administration in rats. *Pharmacol. Rep.* **69**, 1247-1253 (2017). **IF: 2,787**

9. Ferrante, C., Recinella, L., Leone, S., Chiavaroli, A., Di Nisio, C., Martinotti, S., Mollica, A., Macedonio, G., Stefanucci, A., Dvorácskó, Sz., Tömböly, Cs., De Petrocellis, L., Vacca, M., Brunetti, L., Orlando, G. Anorexigenic effects induced by RVD-hemopressin(α) administration. *Pharmacol. Rep.* **69**, 1402-1407. (2017). **IF: 2,787**

10. Stefanucci, A., Novellino, E., Macedonio, G., Dimmito, M. P., Mirzaie, S., Cardoso, F. C., Lewis, R., Zádor, F., Erdei, A., Dvorácskó, Sz., Tömböly, Cs., Benyhe, S., Pieretti, S., Minosi, P., Mollica, A. Design, synthesis and biological profile of mixed opioid agonist/N-VGCC blocker peptides. *New J. Chem.* **42**, 5656-5659 (2018). **IF: 3,201**

11. Recinella, L., Chiavaroli, A., Ferrante, C., Mollica, A., Macedonio, G., Stefanucci, A., Dimmito, M. P., Dvorácskó, Sz., Tömböly, Cs., Brunetti, L., Orlando, G., Leone, S. Effects of central RVD-hemopressin(α) administration on anxiety, feeding behavior and hypothalamic neuromodulators in the rat. *Pharmacol. Rep.* **70**, 650-657 (2018). **IF: 2,787**

12. Stefanucci, A., Macedonio, G., Dvorácskó, Sz., Tömböly, Cs., Mollica, A. Novel Fubinaca/Rimonabant hybrids as endocannabinoid system modulators. *Amino Acids.* **50**, 1595-1605 (2018). **IF: 2,906**

13. Leone, S., Ferrante, C., Recinella, L., Chiavaroli, A., Mollica, A., Tömböly, Cs., Stefanucci, A., Dimmito, M. P., Dvorácskó, Sz., Verratti, V., De Petrocellis, L., Orlando, G., Brunetti, L. Effects of

RVD-hemopressin (α) on feeding and body weight after standard or cafeteria diet in rats. *Neuropeptides*. **72**, 38-46 (2018). **IF: 2,915**

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