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-oxymorphone 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-penty1-1H-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.

The aims of the study presented here were the following:

- 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 \textit{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 \[^{35}S\]GTP\(\gamma\)S functional assay.

• To investigate the permeability of selected bivalent derivatives through the blood brain barrier.

• To test the \textit{in vivo} antinociceptive effects of the \textit{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), ΔPro¹-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 ΔPro¹-Hp(1–7) was labeled with tritium under heterogenous conditions.

Characterization of the novel CB receptor radioligands
[^3H]JWH-018 and[^3H]hemopressin(1-7)
Before their application in radioligand competition assays,[^3H]JWH-018 and[^3H]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}\text{H}]\text{JWH-018}\) and \[^{3}\text{H}]\text{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 \(B_{\text{max}}\) 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}\text{H}]\text{DAMGO}, the DOR selective \[^{3}\text{H}]\text{Ile}^{5,6}\)-deltorphin-2, the KOR selective \[^{3}\text{H}]\text{HS-665}\) and the CB receptor radioligands \[^{3}\text{H}]\text{JWH-018}\) and \[^{3}\text{H}]\text{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 \(\text{Hp(1-7)}, \text{Hp(1-9)}\) and \(\text{RVD-Hp(1-9)}\) to inhibit the binding of \[^{3}\text{H}]\text{Hp(1-7)}\) to rat and CB1 knockout mouse brain membrane homogenate.

**Ligand stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S binding assay}**

The signaling properties of the bivalent compounds and hemopressins were investigated in ligand-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}
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 CONCLUSION

✓ 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, $[^3H]$JWH-018 and $[^3H]$Hp(1-7) were prepared and validated.

✓ It was found that $[^3H]$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 ($B_{max} = 1120 \pm 89 \text{ fmol/mg protein}$). In displacement studies $[^3H]$JWH-018 competed with the classical orthosteric CB receptor ligands but not with hemopressins and opioid ligands.

✓ $[^3H]$Hp(1-7) displayed saturable binding in rat brain membrane and also in a CB1 knockout mouse brain homogenate. The receptor bound $[^3H]$Hp(1-7) could not be displaced by JWH-018, rimonabrant 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 \textit{11} exerted its G-protein activation through the MOR and CB2 receptors, while the agonist bivalent compound \textit{19} exerted its G-protein activation through the MOR and CB1 receptors.

Dimerization of MOR and CB agonists resulted in the agonist bivalent compounds \textit{11} and \textit{19} with antiallodynic activity \textit{in vivo} during the early and late phase.

At spinal level bivalent compound \textit{11} and \textit{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:


Other publications:


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