Rimonabant: a CB_1 receptor antagonist as a direct interactional partner for μ- and δ-opioid receptor

Ph.D. thesis

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LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. ***Zádor, F.***, Ötvös, F., Benyhe, S., Zimmer, A., Páldy, E. **Inhibition of forebrain μ-opioid receptor signaling by low concentrations of rimonabant does not require cannabinoid receptors and directly involves μ-opioid receptors.** *Neurochem Int* 61, 378-88 (2012).

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II. ***Zádor, F.***, Kocsis, D., Borsodi, A., Benyhe, S. **Micromolar concentrations of rimonabant directly inhibits delta opioid receptor specific ligand binding and agonist-induced G-protein activity.** *Neurochem Int* 67, 14-22 (2014).

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Other publications unrelated to this thesis:

Lackó, E., Váradi, A., Rapavi, R., ***Zádor, F.***, Riba, P., Benyhe, S., Borsodi, A., Hosztafi, S., Timár, J., Noszál, B., Fürst, S., Al-Khrasani, M. **A novel μ-opioid receptor ligand with high in vitro and in vivo agonist efficacy.** *Curr Med Chem* 19, 4699-707 (2012).

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Total impact factor: 9.39

INTRODUCTION

Opioid and cannabinoid receptors and their interactions

Both cannabinoid receptor types (CB_1 and CB_2) share many features with all the three classic opioid receptors (μ-, κ- and δ-opioid receptors, abbreviated as MOR, KOR and DOR, respectively). They belong to the G-protein coupled receptor (GPCR) super family and they are mostly coupled to $G_{\alpha i/o}$ type G-proteins inhibiting the presynaptic release of different types of neurotransmitters. Furthermore, in certain forebrain regions, the MOR and CB_1 receptors are co-expressed and co-localized in the same neurons. It has also been shown that these two receptor types are cross-regulated via direct or indirect interactions, and they form heterodimers. Also, DOR and CB_1 allosterically alter each other's activity and heterodimerize, and there is evidence for cross regulation of the KOR and CB_1 receptors too. The interaction between these receptors results many overlapping physiological functions such as nociception, mood regulation, energy and feeding regulation, the regulation of GI motility or the mediation of ethanol effects.

Rimonabant and its interactions with the opioid system

CB_1 receptor is known for having a well-established role in the control of appetite, thus both CB_1 agonists and antagonists have been developed for therapeutic regulation of food intake. Among the CB_1 receptor antagonists, rimonabant, was firstly developed and marketed as an appetite suppressant under the trade name Acomplia®. However, 2 years after its introduction it was withdrawn from the market because of serious psychiatric side effects such as severe depression, anxiety and suicidal thoughts occurred during chronic administration of the drug. Before, as well as after entering rimonabant to the market there were several publications indicating its non-CB_1 receptor related actions, partly its inverse agonistic effects and its dose related side effects. These reports also established numerous unspecific effects at higher concentrations.

Rimonabant can interact with other members of the GPCR family, such as opioid receptors. It has been shown that it can affect the function of MOR through the CB_1 receptor. Additionally, rimonabant reduced opiate self-administration and reward

and suppressed morphine-induced feeding in behavioral studies. There are increasing numbers of investigations reporting a direct effect of rimonabant on opioid receptors. According to previous direct binding affinity measurements, rimonabant is able to bind to all three classic opioid receptors with relatively high, micromolar concentrations. Furthermore, it has been revealed that the opioid system is involved in modulating both the metabolic and mood effects of rimonabant.

AIMS OF THE STUDY

The unspecific behaviour of rimonabant together with its ability to pass through the blood-brain-barrier (BBB) partly caused its dramatic failure as an authorized anorectic drug. Now it is known that the opioid system is also involved in the unspecific actions of rimonabant. Most of the studies examining the actions of CB_1 antagonist on opioid receptors reported the effects to be mediated through CB_1 receptors, but very few studies examined the direct effect of rimonabant on the opioid receptors. Herein we clarify whether rimonabant can directly act on MOR and DOR at the level of ligand-receptor and receptor-G-protein interactions. MOR was chosen because it is one of the most studied opioid receptors, mainly because of its role in pain management. DOR has been studied in a relatively less extent compared to MOR. However, recently there are several studies showing DOR as an emerging therapeutic target.

The following investigations were fulfilled in this study:

- The role of the CB_1 receptor in the binding of rimonabant to MOR
- The binding properties of rimonabant to MOR and DOR in Chinese hamster ovary (CHO) cell membranes overexpressing MOR and DOR
- Docking rimonabant to the active and inactive homology model of MOR to reveal interactions in the ligand-receptor complex
- The effect of rimonabant on DOR mediated G-protein basal activity
- The effect of rimonabant on agonist-stimulated MOR and DOR G-protein activity and the possible role of cannabinoid receptors in this effect

For the binding affinity measurements we performed competition binding experiments with opioid receptor selective radioligands, while the MOR and DOR mediated G-protein activity measurements were carried out in functional [^{35}S]GTPγS binding assays.

METHODS

Radioligand competition binding assays

In radioligand competition binding assays we measured the specific binding of fixed concentrations of MOR and DOR selective radioactive ligands in the presence of increasing concentrations of unlabeled rimonabant. The binding affinity of the unlabeled rimonabant, described by the IC_{50} value, was obtained indirectly from the analysis of the radioligand specific binding using GraphPad Prism 5.0 curve fitting program.

The role of the CB_1 receptor in the binding of rimonabant to MOR was measured in wild type and CB_1 K.O. mouse (both generated on the CD1 background) forebrain membranes using the highly MOR selective tritiated [D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin ([^{3}H]DAMGO). The binding of rimonabant to MOR and DOR was investigated in CHO cell membrane fractions overexpressing rat MORs (CHO-rMOR) and mouse DORs (CHO-mDOR). [^{3}H]DAMGO and the non-selective opioid antagonist [^{3}H]naloxone were applied in the experiments performed in CHO-rMOR cell membranes, while the DOR selective agonist [^{3}H]Ile^{5,6}deltorphin II and the DOR selective antagonist [^{3}H]naltrindole was used in CHO-mDOR cell membranes.

Functional [^{35}S]GTPγS binding assays

During [^{35}S]GTPγS binding assays we monitor the receptor mediated G-protein activation, namely the GDP → GTP exchange of G_α, in the presence of a given ligand concentration. The nucleotide exchange is measured by a non-hydrolysable, radioactive GTP analogue called [^{35}S]GTPγS. By analyzing the specifically bound [^{35}S]GTPγS in the presence of a stimulator ligand added in increasing concentrations, we can determine the maximal stimulation or efficacy (E_{max}) of the receptors G-protein and the potency (EC_{50}) of the stimulating ligand.

The effect of rimonabant on DOR mediated G-protein basal activity was measured in CHO-mDOR and parental CHO (pCHO) cell membranes in the presence of increasing concentrations of rimonabant. The effect of rimonabant on agonist-stimulated MOR and DOR G-protein activity was analyzed in CHO-mDOR cell membranes or

wild type and CB_1 K.O. (CD1 strain) or CB_1/CB_2 K.O. (C57BL/6 strain) mouse forebrain membranes. MOR was stimulated by DAMGO, while DOR was activated by the highly selective DOR agonist DPDPE. Both ligands were applied in increasing concentrations. Rimonabant was added in various concentrations during the receptor stimulation.

Docking experiments

Docking calculations gives the opportunity to gather information about the receptor–ligand complex, such as the estimate of binding energy, the possible intermolecular interactions, orientations or the occurring energy alterations, which are hard to achieve in *in vitro* studies.

The 3D coordinates of the active and inactive conformations of MOR prepared by homology modeling were downloaded from the Mosberg group's webpage. The activated receptor model contained the MOR selective agonist, H-Tyr-c(S-Et-S)[D-Cys-Phe-D-Pen]NH_2 (JOM-6), and the inactive receptor model contained the κ-opioid antagonist, norbinaltorphimine (nor-BNI). Ligands and receptors were prepared for docking using the AutoDockTools program suite and then docked by the program AutoDock4. The receptors were kept rigid in the docking calculations, while the rings of the ligands were either kept rigid or flexible. The calculations resulted in the estimated docking free energies in kcal/mol and the lowest docking free energies obtained were used to rank the ability of the ligands to bind to the receptor. Additionally, the energy balance of the receptor activation process was calculated for each ligand, subtracting the docking energy of the ligand-active receptor complex from that of the ligand-inactive receptor complex ("receptor activation energy"). This value was used to characterize the agonistic-antagonistic nature of rimonabant. The docking poses of rimonabant were analyzed and visualized by the program Chimera.

SUMMARY OF THE RESULTS

Measurements performed at the level of ligand-receptor interaction:

- Rimonabant in **micromolar** concentrations **inhibited** the **agonist** [^{3}H]DAMGO **specific binding independently from the CB_1 receptor** in mouse forebrain, although the inhibition was moderate.

- In CHO cell membranes transfected with MOR and DOR rimonabant **decreased** the **specific binding of the agonists** [^{3}H]DAMGO and [^{3}H]Ile^{5,6}deltorphin II **in micromolar** concentrations. The **specific binding** of the **antagonists** [^{3}H]naloxone and [^{3}H]naltrindole was **inhibited** in **subnanomolar** and **micromolar** concentrations, considering the high and low affinity binding site model. Since CHO cell lines do not express CB_1 and CB_2 receptors physiologically, the observed inhibitory effects of rimonabant on MOR and DOR binding in transfected cell lines are **cannabinoid receptor independent.**

- Docking studies showed that rimonabant can bind to **the inactive state** of MOR with a **lower docking energy** compared with the active state, resulting in **an unfavoured energy balance for the receptor activation**. Similarly, **the antagonist naloxone** also showed an **unfavoured energy balance for receptor activation** allowing to classify **rimonabant as an antagonist**. This is also supported by the presence of a **hydrogen bond** between T218 residue in the TM7 domain and the hydrazide group of rimonabant in the **inactive state**, while **no hydrogen bonds** were observed in the binding pocket of the **active receptor**. This confirms that rimonabant prefers the inactive receptor conformational state.

- The preferred inactive MOR conformation and the more effective inhibition of MOR and DOR antagonist specific binding indicates an **antagonistic behaviour** for rimonabant towards these receptors.

Measurements performed at the level of receptor-G-protein interaction:

- Rimonabant **did not increase** *per se* the **DOR** mediated **basal activity**, thus the **agonistic effect can be excluded**. On the other hand, it decreased the basal activity of **DOR** in CHO-mDOR cell membrane fractions therefore it seemed to behave as an **inverse agonist**. However this effect was **not reversed by the DOR antagonist naltrindole**, and the reduced G-protein basal activity was a**lso observed in pCHO cell lines, which do not express DORs**. Thus the inverse agonistic action of rimonabant was independent from DOR, which confirms another unspecific inverse agonistic effect of rimonabant described previously.

- Rimonabant **inhibited the agonist stimulation of DOR** in CHO-mDOR membrane fractions at **micromolar** concentrations by **decreasing the maximal G-protein activity and agonist potency**.

- **Micromolar** concentrations of rimonabant **inhibited MOR** and **DOR mediated G-protein activity** and **DOR agonist potency** in **mouse forebrain** membrane fractions too, in agonist stimulated G-protein activity measurements. The effects were **independent from both cannabinoid receptors**.

- The inhibitory effects of rimonabant at the MOR- and DOR-G-protein interaction level also **indicate an antagonistic character**, since the **agonist**, and **inverse agonist mechanism can be excluded** according to our results. Furthermore, rimonabant **inhibited MOR** and **DOR mediated maximal G-protein activity** and **DOR potency**.

CONCLUSIONS AND FINAL REMARKS

Our results demonstrate that rimonabant inhibits MOR and DOR function directly at the level of ligand-receptor and receptor-G-protein interaction. Our results also pointed out an antagonistic binding character for rimonabant towards MOR and DOR and this finding was also confirmed by subsequent reports. However the opioid receptors are unlikely possible therapeutic targets for rimonabant because of its relatively low affinity towards these receptors, low dose combined treatment with opioid antagonists have promising therapeutic applications as recently published. Very recently MOR agonist/rimonabant hybrid ligands were constructed, which is also a possible approach for future therapeutic applications. We think that our study may contribute to the development of these hybrid ligands in the future.

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