**Eötvös Loránd University**

Doctorate School in Biology;

Head of school: Dr. Anna Erdei

Neuroscience and Human Biology Ph.D. Program;

Head of program: Dr. László Détári D.Sc.

# *In vivo* pharmacology of cannabinoid type 1 receptor antagonists with special attention on their anxiogenic effects

**Ph.D. Thesis**

Balázs Varga

Supervisor:

Dr. József Haller

Head of the Behavioural Neurobiology Department in the Institute of Experimental Medicine of the Hungarian Academy of Sciences, D. Sc.

Consultant:

Dr. István Gyertyán

MTA-SE NAP B Cognitive Translational Behavioural Pharmacology Group

Gedeon Richter Pharmaceuticals Plc

2016

# 1 Introduction

Since the ancient times, men had been using hemp for industrial, recreational and medical purposes, however, after the Marihuana tax act of the United States proclaimed all cannabis derivatives to illicit drugs in 1937, hemp products disappeared from the American pharmacopeias – and subsequently from other countries' worldwide –, and studying cannabinoids became controversial. However, when the cannabinoid type 1 receptor (CB1) was identified as the one responsible for the main behavioral effects of cannabis in 1990, scientific research gained revived momentum and the endokannabinoid system was characterized in a decade's time. In parallel, *in vivo* physiological and pharmacological studies revealed, that genetic and pharmacologic inactivation of the CB1 receptor in rodents may lead to profound appetite and body weight loss.

By these results, CB1 antagonist development became one of the most competitive areas of pharmacology. Fueled by the exceptionally good translatability of the anti-obesity effects, nearly all the big pharmaceutical companies developed at least one CB1 blocking anti-obesity drug. Some of these compounds reached to phase III clinical trials – involving thousands of patients in total – by the middle of the last decade. The Gedeon Richter Pharmaceutical Plc. joined this competition with its own developmental program in 2005, which led to a patent application just within two years. However, in 2008, it has been concluded based on the clinical studies of the prototypical CB1 antagonist, rimonabant, that the psychiatric side effects – anxiety and depression at first place – can seriously compromise the clinical applicability of CB1 antagonists. Consequently, big pharma companies shut down their developmental programs hastily, and so did the Gedeon Richter Plc. A key question has emerged: is there any way to decrease the incidence of the psychiatric side effects of CB1 antagonists?

# 2 Aims

Within the framework of my doctoral studies, we tried to answer the question, whether it is possible to develop a CB1 blocker, which is free of anxiety related side effects. The experimental work plan consisted of three parts described below.

1.) "**Rimonabant characterization**": we investigated weight loss and side effects inducing properties of the best known (both in animals and humans) prototypical CB1 inhibitor, the diaryl pyrazole structured rimonabant in detail.

We wanted to clarify which tests are suitable for the investigation of the weight loss inducing potential and side effects of the CB1 blockers?

2) We **compared eight CB1 inhibitor compounds** with respect to their weight reducing and anxiogenic potentials using an *in vivo* test cascade compiled on the basis of the rimonabant characterization in order to answer the following questions:

a.) Are there any differences in the main and side effect profiles of the diaryl pyrazole analogs?

b.) Do the effects of the non-diaryl pyrazole structured classical cannabinoids differ from rimonabant analogues?

c.) Is it possible to improve the effect / side effect ratio using non-conventional (partial agonist, neutral antagonist, peripherally acting compounds) CB1 inhibitor mechanisms?

3) In **the CB1 mGluR5 interaction study**, we investigated if the co-administration of a metabotropic glutamate receptor 5 (mGluR5) negative allosteric inhibitor, in our case the MTEP :

a.) Can improve the food intake suppressive effects of rimonabant?

b.) Can influence the anxiogenic effects of rimonabant?

# 3 Materials and Methods

## 3.1 Rimonabant characterization

In our first series of experiment using orally administered rimonabant we investigated which *in vivo* tests can be capable for the characterization of the effects of CB1 antagonist on obesity and anxiety in a human relevant manner. Basic pharmacodynamics dose response relationships were analyzed by the inhibition of the CB1 agonist WIN 55,212-2 induced hypothermia in lean CD1 mice. Within the framework of the anti-obesity effects' profiling, effects on acute food intake, locomotion, metabolic rate and respiratory quotient (RER) were measured by a metabolic cage experiment ("Phenomaster test"), while chronic body weight effects were studied by the diet induced obesity (DIO) test. In both tests C57Bl/6 mice fed with high fat containing diets for 3 months were used. In order to gain a more comprehensive picture about the anti-obesity effects, sub-chronic consequences of the rimonabant treatment were studied not only in dietary obese mice, but also on their low fat diet fed siblings. Moreover relative importance of decreased food intake on weight loss efficacy was also investigated using the so called "paired feeding" protocol, in which mice do not receive active treatment, but their maximum daily intake cannot exceed the former day intake of the rimonabant treated group. After the experimental phase, mice were sacrificed and serum leptin, glucose, cholesterol and triglyceride levels were measured. However before the blood collection, tail suspension (TST) and elevated plus maze tests were performed with the same mice in order to detect potential depressive or anxiogenic effects induced by the former, two-week rimonabant treatment. Subsequently, acute neuropsychiatric symptoms were screened with a toxicological screen method, the Irwin test, up to the 300 mg/kg dose of rimonabant. Based on the results of this, we installed an anxiety test, the ultrasonic vocalization (USV), which is more suitable to measure the effects of stress induced fear and panic susceptibility. In addition, anxiogenic potential was investigated by two other – more trait anxiety sensitive – methods, the Vogel punished drinking test and the elevated plus maze test. Finally, the translatability of the results has been confirmed by food intake tests carried out with identical protocols.

## 3.2 Comparison of the CB1 inhibitors

In the second part of our experimental program, we studied the main and side effect profiles of various CB1 inhibitors using a test cascade compiled from methods, which were validated earlier (see 3.1). The eight CB1 inhibitors investigated here were selected to represent all the approaches deemed to bear developmental-pharmacologic importance by us:

a partial agonist, neutral antagonists and inverse agonists (functional approaches); diaryl-pyrazole and other structured compounds (medicinal chemistry approaches); and peripherally and centrally acting drugs (kinetic approaches). Of course, we could not test all the theoretically potential combinations of the above-mentioned approaches, but, by the selection of prototypic reference compounds, we sought to be able to achieve decisive conclusions about their therapeutic potentials. Five well-known compounds –all tested in clinical studies – have been described among the "classical CB1 antagonists", which stands for drugs ceased to develop in 2008. Among them, two were diaryl-pyrazole structured analogues, rimonabant and surinabant; while three were other structured, ibipinabant, taranabant and otenabant. Among the "non-conventional CB1 inhibitors", which belong to the promising new developmental approaches, four prototypic, yet poorly characterized compounds were investigated, which have not been studied in humans so far. Representatives of partial agonism, centrally acting neutral antagonism, peripherally acting neutral antagonism and peripherally acting inverse agonism were O-1269, VCHSR, LH-21 and JD-5037, respectively. Among these, the first three compounds were rimonabant analogues, while the last one was an ibipinabant analogue. The last two compounds were putatively peripherally selective compounds. Two non-cannabinoid type molecules, the non-CB1 binding ibipinabant enantiomer (+)SLV-319 and the monoaminergic reuptake inhibitor sibutramine were used as positive and negative reference compounds in the obesity related tests.

In order to determine pharmacological potentials of these compounds under standardized conditions, we built up a preclinical test system based on the rimonabant tests results.. *In vivo* central CB1 antagonist potencies were determined by using the inhibition of agonist induced hypothermia, while appetite suppression was measured by fasting induced food intake test. Both tests were carried out using lean CD1 mice. Based on the results of these tests, putatively equally effective doses have been proposed to compare weight loss independent effects (especially the syndrome X related blood markers). Eligible CB1 inhibitors at these equi-effective doses were administered to dietary obese (DIO) C57Bl/6 mice for 2 weeks, while food consumption and body weight were measured daily. At the end of the experimental phase, triglyceride, glucose and cholesterol levels were measured. Subsequently, obesity-dependence of the appetite suppressive effects were studied by a specifically developed "home-cage food intake test" at the equi-effective doses used in the DIO test, administered to obese mice and their lean siblings. To determine side effects, we were bound to the USV test in rats, by which dose-response curves have been recorded for

every compound. Finally, translatability of results between rats and mice was investigated by comparison of dose-response curves from similarly conducted fasting induced food intake tests.

### 3.3 The CB1-mGluR5 interaction

In our third set of experiments, effects of the best-known CB1 and mGluR5 blockers, rimonabant and MTEP, were investigated in rats both one by one and in coadministration in order to study their potential interactions in the areas of anxiety and food intake. The applied methods were basically similar to the formerly used ones, but some modifications had to be made to take the differences of both systems into account: food intake experiments were performed with shorter fasting times, while USV tests were carried out with a stronger shock intensity to be able to properly investigate the anxiolytic effects of MTEP.

Where it was applicable, effects of the compounds were compared by the appropriate statistical analysis (one-way, repeated measures or factorial) ANOVA, followed by a post hoc (usually Tukey – sometimes Duncan) test.

## 4 Results and Discussion

### 4.1 Rimonabant characterization

Rimonabant, applied orally with one hour pretreatment time, inhibited the agonist induced hypothermia in a dose dependent manner. Its least significant dose (LED) was 1 mg/kg, however, the calculated dose of 50% inhibition (ID50%) was as low as 0.41 mg/kg, and it induced 100% inhibition at the 3 mg/kg dose. Anti-hypothermic effect of the 3 mg/kg dose decreased in a time dependent manner, but it was noticeable even after 24 hours Rimonabant *per se* did not influenced body temperature at all.

With respect to the anti-obesity effects, rimonabant decreased food intake in dietary obese mice through 24 hours in the Phenomaster instrument. In parallel, respiratory exchange rate decreased near to the physiological minimum (0.7), while metabolic rate slightly (non-significantly) increased in instead of a compensatory decrease. Locomotor activity did not rise significantly.

Investigating the anti-obesity effects of the two-week subchronic treatment in the DIO test,, rimonabant decreased body weight of the obese mice in a dose dependent manner. Although its LED dose was 10 mg/kg, it decreased body weight compared to the initial

weight by nearly 10% even at 3 mg/kg dose and at 30 mg/kg dose the decrease reached 25%. Decreases of the daily food intake correlated well with daily change in body weights throughout the whole test. In order to get more detailed data, effects of the 10 mg/kg dose were investigated in an additional experiment. During this experiment, the body weight decreasing effect of rimonabant was significant only in case of obese (-15%), but not in low fat diet fed lean mice. In the latter case rimonabant only induced modest (5%) effects, while in the obese paired fed group, the weight decrease (8%) was in between the control and the treated groups. Based on the blood marker levels, determined after the two weeks of treatment, rimonabant significantly decreased the obesity-induced elevations of leptin and glucose levels, however, it did not influence cholesterol levels, and lowered triglyceride levels in obese as well as in lean mice.

DIO mice, used in the above mentioned obesity tests have also participated in subsequent tail suspension or elevated plus maze measurements, in order to investigate the potential depressive or anxiogenic side effects of the former subchronic rimonabant treatments. However no such effects were found. Then, we decided to investigate neuropsychiatric side effects of acute rimonabant using a toxicological profiling method, the modified mouse Irwin test. Its results showed that rimonabant might influence behavioral functions related to motor coordination, touch escape response and reactivity at first place. According to these, two specific experiments the rotarod and the ultrasonic vocalization (USV) tests were performed for the better characterization of potential motor coordination and anxiety related effects:. While rimonabant did not influence motor skills on the rotarod up to 30 mg/kg in mice, it significantly increased the anxiety related vocalization time from the 1.25 mg/kg dose in the USV test in rats. However, no effect of rimonabant was found in two other rat methods measuring trait anxiety, the Vogel punished drinking and the elevated plus maze tests. Finally, in fasting induced food intake tests conducted with identical protocols in rats and mice, rimonabant induced food intake suppression with similar dose response curves, reinforcing the translatability of the anti-obesity effects between rodent species.

Results of hypothermia test confirmed, that rimonabant is orally available, has good pharmacokinetic profile so it is applicable as validating agent. In accordance with literature and clinical data, our results obtained by using obese mice have shown that rimonabant is able to decrease body weight in a dose dependent manner – and this effect is mediated mainly through the loss of food intake. Weight loss is independent from the species studied (rats or mice), but it do depend on the obese phenotype, moreover, it is accompanied by decreases in

respiratory quotient, triglyceride, leptin and blood glucose levels. We were unable to find significant anxiogenic or depressive side effects neither after DIO tests, nor after acute rimonabant treatments in the elevated plus maze or Vogel test, measuring trait anxiety. Though this lack of efficacy is apparently in contrast with the clinical failure caused by the psychiatric side effects of rimonabant, we have to note, that in former preclinical literature reports rimonabant was sometimes anxiogenic, sometimes anxiolytic; and the amount of increases of the side effects seen in the human phase III studies, might be too low to detect in animal tests. However, by the application of the ultrasonic vocalization test, we were able to find a better detectable aspect of the anxiogenic effects of CB1 antagonists. Its results have unequivocally shown, that the anti-obesity and the anxiogenic effects lay within the same dose range.

In conclusion, as the *in vivo* efficacy profile of rimonabant was well characterizable by the application of hypothermia, food intake and DIO test, blood level determination, obesity dependent efficacy and USV tests, thus these experiments opened the possibility to investigate other compounds using the same models as well.

## 4.2 Comparison of the CB1 inhibitors

Using the above mentioned methods, we have compared the efficacy profiles of: two diarylpyrazoles (rimonabant and surinabant); three other, structurally different CB1 antagonists (ibipinabant, taranabant, otenabant), and we tried to describe the effects of non-conventional CB1 inhibitors, like the partial agonist O-1269, the centrally and peripherally acting neutral antagonists VCHSR and LH-21 and the peripherally acting JD-5037.

Rimonabant and surinabant, applied at similarly potent doses, induced weight loss and food intake suppression in similar manner. However their anxiogenic effects – measured by the USV test – were also similar. Yet, among the non-diarylpyrazole CB1 antagonists we did find some differences, like the outstanding pharmacokinetic potential of taranabant, or the inefficacy of otenabant in the ultrasonic vocalization test. Interestingly, irrespective of their structures, all classical CB1 antagonists induced similarly obesity dependent appetite suppression in the home-cage food intake test. In case of the blood-level determinations, we could only find one significant difference, the reduction of triglyceride levels induced by rimonabant treatment. Regretfully, none of the non-conventional CB1 inhibitors were eligible for main/side effect determination. O-1269 and LH-21 induced convulsion, while VCHSR was ineffective in the fasting induced food intake test. Although JD-5037 was also ineffective

on food intake, we still investigated it in the obesity models at doses used in the literature; however, it did not show any activity.

Our results acquired by testing of the classical CB1 antagonists were in good agreement with literature data. The similarity between the effects of the diaryl pyrazoles and the somewhat better risk/benefit ratio of the non-diarylpyrazoles were probable based on literature data. However, neither the generalizability of obesity dependence of the appetite suppression, nor the unique triglyceride level lowering efficacy of rimonabant were predictable. In case of the non-conventional CB1 inhibitors, we found even more differences compared to the literature. Probably the most important ones among these were the convulsive side effects of LH-21 and O-1269 and the inactivity of JD-5037 in the DIO test. Lack of the appetite suppressant effects of VCHSR in the food intake test was also surprising. In conclusion, our results shows that among the classical CB1 antagonist, diaryl pyrazoles have generally poor main/side effect ratio, while compounds with other structures can bear better *in vivo* profile, though side effects might not be completely eliminated with this approach either. However, new, more optimized compounds might be necessary to estimate the risk/benefit ratios of the non-conventional CB1 antagonist approaches, Regretfully, none of the non-conventional CB1 inhibitors were eligible for risk/benefit determination: while O-1269 and LH-21 proved to be toxic, VCHSR and JD-5037 were ineffective.

The novel, original molecule, by Richter "compound 11r", which was identified by the application of the same screening cascade used in the CB1 antagonists comparison, may have overtly similar *in vivo* profile to the two above mentioned two diaryl pyrazoles, yet it distinguished itself by its exceptional cholesterol-level suppressing efficacy. However, after the fall of rimonabant, its development could not be started.

## 4.3 The CB1-mGluR5 interaction

In the last section of our experimental program, we investigated the coadministration of rimonabant and MTEP in food intake and anxiety tests. We have shown, that appetite suppressant efficacies of the two compounds are additive at low doses, but infra-additive at high ranges, while the effect of their combination in the ultrasonic vocalization test was always anxiolytic. Thus, by the combination of the CB1 and mGluR5 antagonism, a truly anxiogenic side effect-free anti-obesity effect could indeed be achieved, however, the coadministration of the two above mentioned compounds did not suppress but enhanced cognitive impairments thus the development of such a combination became impassable.

# 5 Conclusions and New Scientific Achievements

1.) **Based on the rimonabant characterization, we can conclude, that**

*in vivo* main and side effect profiles of the CB1 antagonists can be reliably studied by using CB1 agonist induced hypothermia, fasting induced food intake, diet induced obesity tests; analysis of blood markers, obesity dependence and ultrasonic vocalization.

*Within the framework of rimonabant characterization, we first*

- *described the interaction between the decreased food intake and weight loss in DIO mice*
- *characterized the metabolic effects of drug induced appetite suppression*
- *demonstrated the USV enhancing effects of rimonabant in adult rats.*

**2.) Our results shows that among the classical CB1 antagonists:**

a. main and side effect ratio of diaryl pyrazoles are poor;

b. some of the non-diaryl pyrazole ligands, such as taranabant and otenabant have more favorable main and side effect ratios, but they are not competly free from side effects;

c. In case of the non-conventional CB1 inhibitors, risk/benefit ratios could not be determined, thus development of more optimized compounds is necessary.

*Within the framework of the comparison CB1 inhibitors, we first*

- *described the convulsive effects of LH-21 and O-1269*
- *generalized the obesity dependence of appetite suppression to all CB1 blockers*
- *compared the USV enhancing effects of the CB1 blockers in adult rats*
- *described the efficacy profile of compound 11r*

3.) **Using the combination of rimonabant and MTEP, we first demonstrated, that**

a. their combination at low doses can increase appetite suppression;

b. their combination is anxiolytic even under stressful conditions.

Our studies contributed to the birth of many posters, four articles and a patent. They also established the screening cascade of Gedeon Richter Plc's CB1 antagonist program, and supported the methodical basis for the later founded obesity work group and its drug-combination developing culture. Though this program ended, other CB1 antagonist developments are ongoing worldwide: several academic laboratories are developing new non-conventional CB1 inhibitors, others investigate the efficacy of rimonabant for treatment of more serious diseases than obesity and promising ideas of combining CB1 antagonists with compounds like fluoxetine, MCHR1 antagonists or 5-HT ligands has emerged.

# List of relevant publications

**Posters**

**Varga B**., Szikra J., Kurkó D., Szabó Gy., Fischer J., Kiss B., Nagy J., Gyertyán I: In vitro and in vivo comparison of three CB1 antagonists. FENS, 5th Forum of European Neuroscience; Vienna, Austriaé 8-12 July 2006

**Varga, B**.,Hegyi, É.,Boros, A.,Selényi, Gy., Gyertyán, I. Characterization of rimonabant's effects on body weight and serum lipid parameters using lean, dietary obese and pair-fed mice. EBPS, 13th Biennial Meeting; Rome, Italy; 4-7 September 2009

**Patent**

János Fischer, Attila Szemző, György Szabó, Peter Erdélyi, **Balázs Varga**, Istvan Gyertyán, Judit Szikra, Mónika Vastag; Novel CB1 antagonists and their preparation WO 2008075118 A1 (granted June 26, 2008).

**Publications**

Szabó G, **Varga B**, Páyer-Lengyel D, Szemzo A, Erdélyi P, Vukics K, Szikra J, Hegyi E, Vastag M, Kiss B, Laszy J, Gyertyán I, Fischer J. (2009): Chemical and biological investigation of cyclopropyl containing diaryl-pyrazole-3-carboxamides as novel and potent cannabinoid type 1 receptor antagonists. J Med Chem. 52: 4329-37.

**Varga B**, Kassai F, Gyertyán I (2012): Interactions of CB1 and mGlu5 receptor antagonists in food intake, anxiety and memory models in rats. Pharmacol Biochem Behav. 103:425-30.

Kitka T, Tuza S, **Varga B**, Horváth C, Kovács P. (2015): Differential regulation of metabolic parameters by energy deficit and hunger. Metabolism. 64(10):1235-9.

**Publication under rewision**

**Varga B**, Kassai F, Szabó Gy, Kovács P, Fischer J, Gyertyán I: Pharmacological comparison of conventional and non-conventional cannabinoid receptor 1 inhibitors in rodent in vivo models; *Under revision*

**Publications used as supporting material only:**

Haller J, Mátyás F, Soproni K, **Varga B**, Barsy B, Németh B, Mikics E, Freund TF, Hájos N. (2007): Correlated species differences in the effects of cannabinoid ligands on anxiety and on GABAergic and glutamatergic synaptic transmission. Eur J Neurosci. 25:2445-56.

Mikics E, Dombi T, Barsvári B, **Varga B**, Ledent C, Freund TF, Haller J. (2006): The effects of cannabinoids on contextual conditioned fear in CB1 knockout and CD1 mice. Behav Pharmacol. 17:223-30.

Haller J, **Varga B**, Ledent C, Freund TF. (2004): CB1 cannabinoid receptors mediate anxiolytic effects: convergent genetic and pharmacological evidence with CB1-specific agents. Behav Pharmacol. 15:299-304.

Haller J, **Varga B**, Ledent C, Barna I, Freund TF (2004): Context-dependent effects of CB1 cannabinoid gene disruption on anxiety-like and social behaviour in mice. Eur J Neurosci. 19:1906-12.