# STUDY OF PSYCHOSIS AND ANTIPSYCHOTICS BY NEUROCHEMICAL AND BEHAVIOURAL PHARMACOLOGICAL METHODS

***PhD Theses***

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## INTRODUCTION

The prevalence of schizophrenia is about 1% in the population and about 10% of these patients commit suicide attempt in their lifetime. In the etiology of the disorder there are genetic and environmental risk factors. The nearly published (2013) DSM-V provides a significant support in the therapeutic treatment of schizophrenia, due to the novel diagnostic criterias [*Heckers et al., 2013; Bhati, 2013*].

There are three types of symptoms in schizophrenia, positive, negative and cognitive [*Kapur and Mamo, 2003; Lieberman et al., 2005*]. The first generation of antipsychotics had therapeutic effect on the positive symptoms. Second generation antipsychotics showed considerable influence on the negative symptoms as well with lower risk of side effects. The treatment of cognitive symptoms of schizophrenia is still unsolved.

There are three main neurochemical theories of schizophrenia, the dopamine (DA), the serotonin (5-HT) and the glutamate theory. Dopamine theory is the earliest and most evidence based one. According to the DA theory the essential effect of the antipsychotics is the antagonism of $D_2$ receptors and the central DA system is regionally unbalanced in schizophrenic patients [*Hertel et al., 1995*]. Serotonin theory was emerged with the appearance of clozapine and the focus moved to the antagonism of 5-$HT_{2A}$ receptors [*Meltzer et al., 1989, 2003*]. Clozapine proved to be effective in the treatment of the negative symptoms of schizophrenia without the extrapyramidal side effects (EPS) of the first generation antipsychotics. Further developments turned to the Multi-acting Receptor Targeted Antipsychotics (MARTA). According to the glutamate theory of schizophrenia the hypofunction of NMDA receptor has a major role in the patomechanism of the disorder, especially in the development of negative and cognitive symptoms [*Javitt et al., 1987, 2004, 2005*]. NMDA receptor function could be modulated by the glycine level in the brain which is regulated by the glycine transporters (GlyT) [*Dingledine et al., 1990*]. Glycine transporter inhibitors were developed to potentiate NMDA receptor function through an elevated level of glycine concentration in the synaptic cleft.

GlyT inhibitors were a promising new therapeutic way in the development of antipsychotics, although they haven't proved to have enough efficacy in the therapies. Development as an adjuvant therapy could be a potential use of these molecules in the future.

Research and development of compounds acting on the disorders of Central Nervous System are the traditional research field of Egis PLC, including development of neuroleptics. The subject of the dissertation is the pharmacological study of Egis-11150, an original antipsychotic [*Gacsályi et al., 2013*], risperidone, a glycine transporter inhibitor ORG-24461 and combination of ORG-24461 and risperidone.

## OBJECTIVES

Objectives of the dissertation:

1. Study of the receptor profile of Egis-11150, characterization of the agonist/antagonist activity of the molecule in functional *in vitro* assays.
2. Comparison of the receptor profile of Egis-11150 with that of risperidone and ORG-24461 on the basis of own experimental and published results.
3. Studies of the efficacy of Egis-11150 and risperidone in *in vivo* animal models of psychosis.

4. Comparison of Egis-11150, risperidone and ORG-24461 in *in vivo* experimental schizophrenia models based on published and own measurements' results.
5. Studies of Egis-11150 and risperidone in experimental models of cognition.
6. Studies of combination of risperidone and ORG-24461 in *in vivo* animal models of psychosis.
7. Studies of combination of risperidone and ORG-24461 in experimental models of cognition.
8. Studies of the effects of Egis-11150, risperidone and ORG-24461 on the striatal dopamine, DOPAC, HVA, glutamate and glycine levels with microdialysis technique.
9. Studies of the combination of risperidone and ORG-24461 on the striatal dopamine, DOPAC, HVA, glutamate and glycine levels with microdialysis technique.

## METHODS

### Receptor binding assays

Measurements were performed in $10^{-5}$ and $10^{-7}$ M concentration on more than 50 receptors with Egis-11150. Risperidone and ORG-24461 were studied on 11 receptors (NMDA, 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_{2C}$, 5-$HT_6$, 5-$HT_7$, $\alpha_1$, $\alpha_2$, $\beta$, $D_1$ és $D_2$) in the same concentrations. If inhibition of the binding affinity reached the 50% in $10^{-7}$ M concentration, $K_i$ value was determined.

### [$^3$H]glycine uptake in rat cerebral cortex synaptosomes

Synaptosomal $P_2$ fraction was prepared as described previously [*Szasz et al., 2005*]. The uptake was initiated by adding 0.3µM [$^3$H]glycine (specific activity: 14 Ci/mM). To achieve 0.3 µM glycine concentration unlabeled glycine was added to an aliquot of [$^3$H]glycine. Specific uptake was calculated by the subtraction of nonspecific uptake from the total uptake value. Each drug concentration was tested in three parallels of samples. Protein content of the preparation was determined by the method of Lowry, using CuEDTA [*Lowry et al., 1951*]. The value of $IC_{50}$ was calculated using non-linear regression.

### In vivo microdialysis in conscious rats

Surgery was performed under pentobarbital (60mg/kg ip.) anesthesia in stereotaxic frame (*David Kopf Instruments, USA*). A rostrocaudal incision was made above the sutura sagittalis to expose the surface of the skull. According to the localization [*Paxinos and Watson, 1998*] AP (antero-posterior): -0.4 and ML (medio-lateral): +3.5, the skull was drilled. The guide cannula was implanted to the needed depth, DV (dorso-ventral): -4.0 and secured. On the following day of the surgery the 2 mm length microdialysis probe was inserted through the guide cannula into the striatum. After 2 hours equilibration period 10 fractions were collected, 30 min long each.
After the collection of the fourth fraction risperidone (1 mg/kg), Egis-11150 (0.1 mg/kg), ORG-24461 (10 mg/kg), or risperidone and ORG-24461 combination was administered intraperitoneally (ip.). Cathecolamines and their metabolites [dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] were measured immediately after the collection of the samples [*Adams and Marsden, 1982*], and were frozen on -80 °C. The concentration of extracellular amino acids were measured from the frozen samples [*Rowley et al., 1995*]. Later the brains were sliced coronal to determine the exact position of the probe.

**Apomorphine induced stereotypy and climbing in mice**

Egis-11150, risperidone and vehicle (0.4% MC) were administered orally (p.o.). 30 min after the administration, animals were placed individually into the experimental chamber for habituation. After 30 min habituation animals were administered with 1 mg/kg apomorphine subcutan (sc.). The measurement of stereotype behaviour began immediately after the apomorphine treatment and lasted for 25 min. Stereotype was scored from 0-4. Climbing behavior was scored in an "all or none" manner 15 min after apomorphine administration for 10 min. $ID_{50}$ values for stereotypy were calculated by linear regression analysis using % inhibition.

Climbing frequency was calculated for each group. The results of control group was considered as 100%. $ED_{50}$ value was computed from the data of dose-effect relationship by the method of Litchfield and Wilcoxon [*Litchfield and Wilcoxon, 1949*].

**Catalepsy inducing effect in rats**

The cataleptogenic effect was studied according to the slightly modified method of Morpurgo [*Morpurgo, 1962*]. Egis-11150, risperidone and vehicle (0.4% MC) were administered orally 60 min before the experiment. Both fore-paws of the rat were placed firstly on a 3.5cm tall and secondly on a 9.5 cm tall rubber stopper one by one. If the animal does not remove the paw from the stopper during 10 seconds it is evaluated as catalepsy. The measurement of catalepsy was performed every 30 min for four hours. Catalepsy was graded from 0 to 1. The score maximums were determined for every group, which were used for the calculations of % effects. The highest score (3) multiplied by number of animals/group was considered as 100 %. $AD_{50}$ (AD=active dose) values were determined from the % values by linear regression.

**Spontaneous motor activity in rats**

The rats were placed for 15 min in the middle of the experimental boxes after the administration of MK-801 sc., risperidone p.o., Egis-11150 and ORG-24461 ip., 30 or 60 min before the experiment. Spontaneous activity was recorded as a consecutive break of 2 photo beams, spaced at 190 mm from the walls and each other, and placed 40 mm above the floor.

**MK-801 induced hypermotility in rats**

The experiment was performed in the same apparatus as the spontaneous motor activity. Egis-11150 and risperidone were administered ip., MK-801 sc. in the same time, 30 min before the experiment, ORG-24461 was administered ip. 60 min before the performance of the study. In the case of the combination (risperidone+ORG-24461) ORG-24461 was administered 30 min before the risperidone and MK-801 administration. The experiment was performed 30 min after the treatment with risperidone and MK-801. The measurement lasted for 15 min.

**Phencyclidine (PCP) induced disruption of prepulse inhibition (PPI) in mice**

Studies were performed in the TSE startle response system (TSE GmbH, Germany). Egis-11150 and risperidone were administered ip., PCP sc. 30 min, ORG-24461 60 min before the experiment. In the combination study (risperidone+ORG-24461), ORG-24461 was administered ip. 60 min, risperidone ip. and PCP sc. 30 min before the measurement. During the experiment the background noise was 65dB, pulse was 110dB. During the 5 min acclimatization period, the background noise was only presented. This was followed by five trials (110dB) consisting of pulse-alone trials (habituation

period), than 60 trials were used to assess PPI. Levels of prepulse inhibition were determined by the following formula: 100-[(prepulse/no prepulse)×100]. Data were analysed using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons.

**Procognitive efficacy in novel object recognition test in rats**

The test apparatus was a black Perspex box with sawdust bedding. Metal triangular (8.5×5×14 cm) and rectangular prisms (5×5×14 cm) were used as objects to be discriminated. On day 1 the animals were allowed to explore test apparatus without objects for 150 s. 24 h later (sample trial, T1) two identical objects were allowed to be explored for a maximum of 5 min. The rat was considered to explore the object when its nose was towards the object at a distance not exceeding 2 cm while sniffing and/or touching the object. The criterion for inclusion in the study was that during the period of exploration both objects had been explored for at least 10 s - once this was fulfilled, the sample trial was terminated by moving the subject to its home cage. Retrieval was examined 24 h after T1 (choice trial, T2) or 15 min after T1 in the studies with MK-801 induced cognitive deficite.: the animals were allowed to explore a new and a familiar object for 4 min. Drugs were administered on day 2 before acquisition, orally 60 min, ip. and sc. 30 min before T1. In the MK-801 interaction study ORG-24461 was administered 30 min before MK-801 treatment. In the combination study (risperidone+ORG-24461) risperidone was administered ip. in the same time as MK-801 sc. The discrimination index DI=(N-F)/(N+F), where N and F are the total amount of time spent exploring the novel and the familiar object during T2, respectively, was calculated and statistically analysed by one-way ANOVA followed by Dunnett's test. In the MK-801 interaction study the MK-801 group was compared by Student-t test to the vehicle treated group. In this case the drug treated groups were compared to the MK-801 treated group, statistically analysed by one-way ANOVA followed by Dunnett's test. In the combination study all groups were compared to each other by Tukey's test.

**RESULTS (THESES)**

**Receptor binding**

Egis-11150 showed high affinity to $ADR_{\alpha 1}$ ($K_i$ = 0.5 nM), $ADR_{\alpha 2c}$ ($K_i$ = 8.6 and 13 nM), 5-$HT_{2A}$ ($K_i$ = 3.2 nM), 5-$HT_7$ ($K_i$ = 8.4 and 9.9 nM) receptors, moderate or low affinity to $ADR_{\alpha 2a}$ ($K_i$ = 93 and 141 nM), $D_1$ ($K_i$ = 370 and 380 nM), $D_2$ ($K_i$ = 120 nM), $D_3$ ($K_i$ = 370 and 380 nM) and $D_4$ ( $K_i$ = 25 and 110 nM) receptors.

Risperidone showed high affinity to $ADR_{\alpha 1}$ ($K_i$ = 1.6 nM), 5-$HT_{2A}$ ($K_i$ = 0.5 nM), 5-$HT_7$ ($K_i$ = 9.9 nM) and $D_2$ ($K_i$ = 3.4 and 6.7 nM) receptors, moderate affinity to $D_1$ ($K_i$ = 150 nM) receptors.

ORG-24461 didn't show affinity to any of the studied receptors.

Egis-11150 inhibited all physiological agonists of the receptors in the functional assays. Like risperidone, Egis-11150 displayed inverse agonist properties on 5-$HT_{7A}$ receptors.

**[$^3$H]glycine uptake in rat cerebral cortex synaptosomes**

ORG-24461 inhibited the high-affinity [$^3$H]glycine uptake in rat cerebral cortex synaptosomal preparation. Risperidone exhibited no inhibitory potency. $IC_{50}$ values: ORG-24461: $1.3+0.1\times10^{-7}$M and risperidone: $>5.0\times10^{-5}$M.

**In vivo microdialysis studies in conscious rats**

Risperidone (1mg/kg ip.) elevated DA and its metabolites (DOPAC and HVA) levels in rat striatum and it didn't influence the extracellular glycine and glutamate levels. Egis-11150 (0.1mg/kg ip.) increased selectively the DA concentration in rat striatum, DOPAC, HVA and amino acids concentrations were not changed. ORG-24461 (10mg/kg ip.) reduced DA concentration, whilst DOPAC and HVA levels were not changed. ORG-24461 caused a 2.5-fold increase in extracellular glycine concentration.

Risperidone (1mg/kg) and ORG-24461 (10mg/kg) in combination didn't influence DA level in striatum, DOPAC and HVA concentrations were slightly increased. Both glycine and glutamate levels were increased after the administration of the combination.

**Apomorphine induced stereotypy, climbing and catalepsy**

Egis-11150 and risperidone inhibited the apomorphine induced climbing (Egis-11150: 0.06 mg/kg, risperidone 0.02 mg/kg). Risperidone inhibited apomorphine induced stereotypy in a magnitude lower dose (0.08 mg/kg) than Egis-11150 (0.2 mg/kg). Risperidone induced catalepsy in lower doses ($AD_{50}$= 1.3 mg/kg) compared to Egis-11150 ($AD_{50}$= 8.6 mg/kg). ORG-24461 showed no efficacy in apomorphine induced climbing, stereotypy and in catalepsy model.

**Spontaneous motor activity and MK-801 (0.1mg/kg sc.) induced hypermotility in rats**

Risperidone (0.03mg/kg po.) significantly reduced the normal activity of the animals, and it could reverse the MK-801 induced hypermotilty only in higher doses 0.1; 0.3 and 1 mg/kg. Egis-11150 inhibited the MK-801 induced hypermotility in a magnitude lower dose (0.03mg/kg ip.) than the spontaneous activity. ORG-24461 reduced the spontaneous motor activity in 10 and 30mg/kg ip. doses and it could reverse the MK-801 induced hypermotility only in 30 mg/kg.

Risperidone (0.03mg/kg ip.) and ORG-24461 (3mg/kg ip.) in combination significantly decreased the MK-801 induced hypermotility.

**Phencyclidine (PCP) (5mg/kg sc.) induced disruption of prepulse inhibition (PPI) in mice**

Risperidone (0.1mg/kg ip.) and EGIS-11150 (0.03mg/kg ip.) significantly inhibited the effect of PCP in the PPI model. ORG-24461 (1-3-10 mg/kg ip.) could not reverse the PCP disruption. ORG-24461 (1mg/kg ip.) in combination with risperidone (0.03mg/kg ip.) in themselves ineffective doses showed significant efficacy with reversing the PCP disrupted PPI.

**Procognitive efficacy in novel object recognition test in rats**

MK-801 significantly (0.1mg/kg sc.) and dose dependently decreased the discrimination index (DI) in the novel object recognition test. Single administration of risperidone was not effective in this model, although it could reverse in 0.01 and 0.03 mg/kg doses the MK-801 (0.1mg/kg sc.) induced cognitive deficit (DI). Single administered Egis-11150 (0.1 és 0.3mg/kg p.o.) proved to be procognitive in this model. ORG-24461 (1mg/kg ip.) and risperidone (0.1mg/kg ip.) in combination significantly inhibited the MK-801 induced cognitive deficit. The effect of combination was more than 2-fold stronger compared to the single administration of the molecules.

**DISCUSSION**

The antagonist properties of Egis-11150 on α1, 5-$HT_{2A}$, $D_1$-$D_4$, 5-$HT_7$ receptors may be relevant in the efficacy in positive, negative and cognitive symptoms. According to our results Egis-11150 has more favourable receptor binding profile compared to the available antipsychotics on the market. ORG-24461 didn't show efficacy to the studied receptors. However, elevation of glycine concentrations in the vicinity of impaired NMDA receptors may be responsible for its antipsychotic effect. Based on the receptor binding studies of risperidone, (high affinity to $D_2$ and 5-$HT_{2A/2C/7}$ receptors), could be administered in combination with GlyT inhibitors without the enhancement of dopaminergic side effects (extrapyramidal and endocrine).

The influence of Egis-11150 on the studied neurotransmitters is different from that of risperidone. DA level was significantly elevated similar to risperidone in rats striatum, although DOPAC and HVA levels were not modified, probably due to its lower $D_2$ receptor affinity. The levels of amino acids were not influenced by Egis-11150 similarly to risperidone, suggesting that the molecule has no direct effect on the glutamatergic system.

ORG-24461 significantly reduced the DA level in the rat striatum, which can be attributed to its potentiating effect on the NMDA receptor, facilitating GABA release thus inhibiting the striatal DA efflux [*Javitt et al., 2005; de Bartolomeis et al., 2005*]. In combination with risperidone ORG-24461 diminished the dopamine elevation effect of risperidone, whilst the gylcine level is remained increased. The surprising effect of the combination was the elevation of glutamate concentration in the rat striatum [*Nagy et al., 2010*]. According to our results the glycine potentiated glutamate efflux in the presence of GlyT1 inhibitor was intensified if the inhibition of glutamatergic axon terminals blocked due to the $D_2$ receptor blockade of the antipsychotic. Consequently the blockade of $D_2$ receptors on the glutamate axon terminals by risperidone and the inhibition of GlyT1 by ORG-24461 may enhance the striatal glutamate efflux. We may conclude that the simultaneous administration of GlyT1 inhibitor and an antipsychotic probably could positively influence the dopaminerg/glutamaterg balance in schizophrenia.

Egis-11150 similarly to risperidone inhibited the apomorphine induced climbing in low doses, while its effect in stereotypy was in a magnitude higher dose. The catalepsy inducing potential of Egis-11150 was also lower compared to risperidone, predicting the lower potential to elicit extrapyramidal side effects. ORG-24461 had no effect in apomorphine interaction models and in catalepsy.

Risperidone decreased the MK-801 induced hypermotility in the dose range where the spontaneous motor activity was reduced. Egis-11150 contrary to risperidone could reduce the hypermotility in lower dose than its inhibition effect in the spontaneous motor activity. Egis-11150 has a potential direct therapeutic effect targeting the MK-801 induced disturbance. ORG-24461 similarly to risperidone, inhibited the spontaneous motor activity in lower dose than the hypermotility. In combination with risperidone in themselves ineffective doses, the combination had significant effect on hypermotility, the coadministration enhanced the single efficacy of both molecules.

The PCP disrupted PPI was significantly inhibited by Egis-11150 and risperidone as well. ORG-24461 had no effect on PCP disruption in the PPI model. In combination with the ineffective dose of risperidone it caused a significant reduction of the PPI disruption.

Egis-11150 had significant remarkable effect in low doses in the novel object recognition test. Risperidone had no effect on the normal cognitive performance, it could only reverse the amnestic effect of MK-801. In the efficacy of Egis-11150 its higher blood-brain barrier penetration also may play a role [*Gacsályi et al., 2013*]. ORG-24461 showed slight but significant effect in the MK-801 induced cognitive deficit,

however in combination with risperidone the effect was strengthened remarkably. This effect is in line with results of microdialysis, PCP and other MK-801 interaction studies and serves further evidences to the potential therapeutic advances of the combination.
In conclusion, receptor binding character of the Egis-11150 is nearly similar to the second generation antipsychotics, closer to clozapine, without its anticholinergic side effects. Egis-11150 showed significant antipsychotic activity, and it had remarkable procognitive effect in the rodent models. The results predict more beneficial therapeutical profile compared to risperidone.
The combination of risperidone with ORG-24461 probably could be more advantegous comparing to the present clinical monotherapies for all the three symptoms of schizophrenia.
From this point of view it would worth to compare the combination of Egis-11150 and ORG-24461, or Egis-11150 and other GlyT1 inhibitors, developed by Egis PLC in neurochemical and behaviour pharmacological models.[*Harsing et al., 2015*].

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