

# Comparative analysis of anti-toxoplasmic activity of antipsychotic drugs and valproate

Running title : Anti-toxoplasmic activity of antipsychotics and valproate.

Guillaume Fond<sup>a</sup>,MD; Alexandra Macgregor<sup>b</sup>, MD; Marion Leboyer<sup>a</sup>, MD PhD, Ryad Tamouza<sup>c</sup>, MD PhD, Nora Hamdani<sup>a</sup>,MD PhD; Alexandre Meary<sup>a</sup>, MD PhD, Jean-Francois Dubremetz<sup>d</sup>, PhD.

<sup>a</sup> *Université Paris Est-Créteil, Pôle de psychiatrie du Groupe des hôpitaux universitaires de Mondor, INSERM U955, Eq Psychiatrie Génétique, Fondation FondaMental Fondation de coopération scientifique en santé mentale, F-94000 France*

<sup>b</sup> *INSERM U1061, Service Universitaire de Psychiatrie Adulte, Hôpital La Colombière/CHRU de Montpellier, 39 avenue Charles Flahault, F-34000, France*

<sup>c</sup> *Jean Dausset Dept. & INSERM UMRS 940, Hôpital Saint Louis, Paris, F-75000, France*

<sup>d</sup> *UMR 5235 CNRS, Université de Montpellier 2, Place Eugène Bataillon, F-34000, France.*

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Corresponding author

Dr Guillaume Fond

Pole de psychiatrie

Hôpital A. Chenevier, 40 rue de Mesly, Créteil F-94010

tel (33)1 78 68 23 72 Fax (33)1 78 68 23 81

guillaume.fond@gmail.com

Abstract.

Recent studies have shown a strong link between *Toxoplasma gondii* infection and psychiatric disorders, especially schizophrenia and bipolar disorders (odd ratio  $\approx 2.7$  for each disorder). Antipsychotic drugs and mood stabilisers may have anti-toxoplasmic activity that potentially may be associated with better effectiveness in these disorders, but previous results have been few in number and conflicting. We therefore sought to determine which daily-prescribed antipsychotics and mood stabiliser have the best anti-toxoplasmic activity during the development phase of the parasite.

In the present study, we examined the effects of commonly used antipsychotic drugs (amisulpride, cyamemazine, fluphenazine, haloperidol, levomepromazine, loxapine, olanzapine, risperidone, tiapride) and one mood stabilizing agent (valproate) on toxoplasmic activity.

We replicated that fluphenazine has a high anti-toxoplasmic activity, but it does not seem to be a phenothiazine specific class-effect: indeed we found that another first generation antipsychotic, zuclopenthixol, has a high anti-toxoplasmic activity. Valproate, tiapride and amisulpride have no anti-toxoplasmic activity on parasite growth, and the other antipsychotic drugs showed low or intermediate antitoxoplasmic activity.

As it is not possible to know the intracellular concentrations of antipsychotics in the brain, further clinical studies are warranted to determine if these in vitro findings have potential implications in treatment of toxo-positive patients with schizophrenia. These findings may be potentially relevant for the choice of the first-line antipsychotic drug or mood stabiliser in previously infected patients.

Keywords : *Toxoplasma gondii*, antipsychotic, mood stabiliser, schizophrenia, calmodulin, phenothiazine

Background. The implication of infectious events in the development of major psychosis has recently gained increasing attention (see for review, [2, 3, 7]). Rubella, herpes simplex virus (HSV), cytomegalovirus (CMV), *Toxoplasma gondii* (*T. gondii*), and other infections have been shown to be potent disrupters of fetal neurodevelopment leading to abnormalities of brain and behavior, including psychiatric disorders. In this context, one of the most studied link between a pathogen and psychiatric disorders concerns the association between *T. gondii* and schizophrenia [1, 5, 11, 17, 18, 22, 23, 28, 29] but also recently in bipolar disorders [9].

Toxoplasmosis is a major public health concern: it is the most common protozoan parasite infection in the western developed countries, it concerns one third of the global human population [14]. All vertebrates can be infected as intermediate hosts. Two types of infection have been described in humans: congenital infection (transmission from mother to child during pregnancy) and acquired infection, the subject contaminates itself orally (by undercooked meat from infected animals or parasite-contaminated vegetables) or through contact with infected cats, cats being the definitive host of the parasite [6]. The parasite, after crossing the intestinal barrier, infects all types of nucleated cells by forming an intracellular vacuole. This intracellular parasite alters the expression of host cell genes (including brain cells) and persists in the form of cysts, which can reactivate and release parasites by neospread throughout the host, depending on its immune status. Therefore the parasite exists in two forms in the human body: tachyzoites are found in the acute phase of infection, and bradyzoites that are present in the cysts during chronic infection. Medicine currently considers clinically asymptomatic latent infection as unimportant in immunocompetent individuals [27]. Several studies have shown however behavioral changes induced by infection in animals: *Toxoplasma* is an obligate intracellular parasite capable of infecting 30% of microglial cells and 10% of neurons and astrocytes among rats [4]. The tachyzoites manipulate the cells: they alter the expression of genes coding for apoptosis, anti-microbial functions and immune cell maturation. Again in mice, chronic infection with *Toxoplasma gondii* causes an increase of 14% of brain concentrations of dopamine [21], a neurotransmitter involved in schizophrenia in humans [20]. It was further demonstrated that treatment with a dopamine reuptake inhibitor suppressed exploratory behavior in infected male mice whereas it had the opposite effect in controls, suggesting lasting changes in response to dopaminergic stimulation in infected rodents [20]. To our knowledge, the mechanism behind this difference has not been elucidated yet in the literature.

The contextual link between *Toxoplasma* infection in humans and psychiatric disorders (in particular schizophrenia) can be summarized as follows: (i) proven *T. gondii*'s neurotropism and its impact on dopamine pathway [19], (ii) shared epidemiological characteristics between *Toxoplasma* exposure and schizophrenia such as urban living, (iii) parallel increase in *T. gondii* infection and incidence of psychosis (especially schizophrenia) in various populations (USA, Turkey, Iran) [2, 24]), (iv) a significantly high levels of antibodies to *T. gondii* in maternal sera whose offspring(s) subsequently develop schizophrenia later in life [26] and (v) a 2.73 fold increase in overall odds of *T. gondii* seropositivity for schizophrenia measured in a meta analysis [22], the same result has been recently found in bipolar disorders [9]. It has been recently hypothesized that *T. gondii* may reactivate human endogenous retroviruses (HERV) that may lead to brain immuno-inflammation and then mental disorders [10, 16]. It may then be advantageous to choose an antipsychotic and/or mood-stabilising molecule having anti-toxoplasmic properties in patients that have been previously infected by *Toxoplasma*.

However, conflicting results have been reported on anti-toxoplasmic activity of psychotropic drugs. In particular, Holfels et al. [12] studied the *in vitro* effects of the calmodulin inhibitor trifluoperazine hydrochloride, as well as other antimicrobial agents on *T. gondii* tachyzoites. Trifluoperazine hydrochloride was not inhibitory at lower concentrations, but higher concentrations were toxic to cell cultures *in vitro* and therefore their assay could not be used to assess its effects. Nine years later, Jones-Brando and collaborators [13] found that haloperidol had the highest therapeutic index (18.4), followed by 9-OH-risperidone, the principal metabolite of risperidone (6.7), and fluphenazine (5.1). The other antipsychotics such as chlorpromazine (TI = 2.3) had modest therapeutic indices.

The aim of the present study was thus to determine which antipsychotics or mood stabilizers prescribed in daily practice have the best anti-toxoplasmic activity during the development phase of the parasite. The present study is innovative because it tests one mood stabilizing drug on the development phase of the parasite, and 5 antipsychotics never tested.

#### Material and methods.

Cells and parasites. Parasites were maintained by serial passage in human foreskin fibroblasts grown in Dulbecco's modified Eagle medium (DMEM) (GibcoBRL, <http://www.invitrogen.com>) supplemented with 10% fetal calf serum (FCS) and 2 mM

glutamine. Tachyzoites of the RH strain of *T. gondii* were used throughout the study. Intracellular tachyzoite multiplication was measured on HFFs plated on 12-mm coverslips in 24-well plates and fixed 16 h after infection by equal numbers of freshly released tachyzoites. Infection was performed by incubating a parasite suspension in normal culture medium for 4h on the monolayers, then the wells were washed with medium and a dilution in culture medium of the drug to be tested was added to the well for the duration of the experiment. Coverslips were then fixed with methanol and stained with eosine-methylene blue (RAL 555) and then mounted permanently (Pertex; Microm Microtech France). In all cases, the mean number of parasites per vacuole in the control was in the 4-8 ranges.

Drugs and range testes. All antipsychotics and mood stabilizers used in daily practice in France were tested, i.e. amisulpride, cyamemazine, fluphenazine, haloperidol, levomepromazine, loxapine, olanzapine, risperidone, tiapride, valproate and zuclopenthixol. In all cases, the commercial soluble formulation was used, and diluted in the culture medium. Growth inhibition was measured as the ratio between parasite number per vacuole in the drug treated versus untreated cultures. In an initial screening (Table 1), fields were randomly selected, and the numbers of parasites per vacuole were counted using a 40x objective in ten fields per coverslip, with three coverslips per assay.

In a second phase, we chose the antipsychotic which showed the highest anti-toxoplasmic activity in the screening phase, to determine precisely its IC<sub>50</sub> using 3 independent experiments. Data were analysed using GraphPad/Prism4, to produce a non-linear regression curve reporting the ratio of intracellular growth versus control to the drug concentration. The IC<sub>50</sub> was calculated from the three replicates with a confidence interval of 95%. We also chose fluphenazine as a positive control, and haloperidol for which results in the literature are contradictory.

## Results.

An initial single screening involving all drugs diluted in the range 1-50  $\mu$ M was performed and the results are reported in table 1. We differentiated the specific anti-toxoplasmic activity from the general toxic effect, which entirely destroy the cell monolayer (“toxic”) or cause obvious damages (i.e. pycnotic or loosely attached cells) due to drug exposure (“subtoxic”).

Amisulpride, tiapride and valproate did not have inhibitory activity in the assay at 10 or 50  $\mu$ M. Among the others, zuclopenthixol appearing to be the most efficient after fluphenazine was further explored. Haloperidol having been reported before to produce contradictory

results was also analyzed. The fluphenazine assay was performed once, as a control; since the result was similar to published data (IC 50 3.5  $\mu$ M)[8] it was not replicated.

Zuclopenthixol showed a high anti-toxoplasmic activity with an IC 50 of  $8\pm 1,8 \mu$ M (Table 2, Figure 1). The IC 50 of haloperidol was beyond the range of concentration tested (0 to 20  $\mu$ M) and could not be estimated.

## Discussion

Our results are potentially important to improve our ability to prescribe antipsychotics or mood stabilizers based on aetiological pathways. Choosing psychotropic drugs according to their anti-toxoplasmic activity might in the future improve the efficacy of these treatments and thus the prognosis of toxo-positive patients. The number of tachyzoites, the solvents, the time of addition of drug vs. parasite and the length of time post-infection until the assay was stopped, were all different in previous studies[8, 12, 13]. As we chose to focus on the development phase of the parasite, we chose to reproduce Goodwin et al's methodology[8]. We confirmed that fluphenazine has a very good antitoxoplasmic activity, but also that zuclopenthixol, another first-generation antipsychotic drug, has a high activity against the parasite development (with an IC 50 of 8  $\mu$ M), which invalidates the previous hypothesis of a phenothiazine specific class effect (zuclopenthixol belongs to the thioxanthene class)[8]. We also confirmed Goodwin et al findings about haloperidol that has no antitoxoplasmic activity in the 1-10  $\mu$ M range.

Moreover, we performed a preliminary assay on several antipsychotics that have never been reported before (amisulpride, cyamemazine, loxapine, levopromazine,tiapride) which is important for further studies on the in vivo anti-toxoplasmic activity of antipsychotics in schizophrenia. Amisulpride, a second-generation antipsychotic, showed no anti-toxoplasmic effect, whereas cyamemazine levopromazine and loxapine (first generation antipsychotics), frequently prescribed in treatment of acute psychotic agitation, have intermediate anti-toxoplasmic activity. As we did not have any soluble form at the moment of the experiments for clozapine, it was not tested, but Goodwin et al. found no anti-toxoplasmic activity for this antipsychotic drug [8].

Another finding of interest is that valproate, a frequently prescribed mood stabilizer in bipolar or schizo-affective disorders, has no activity on parasite development, whereas it was found to have high anti-toxoplasmic activity on parasite invasion [13]. Given that invasion is likely a more elusive target than development; valproate may probably be less efficient as anti-toxoplasmic agent in major psychiatric disorders.

Limits. None of these drugs, as far as we know, affect the tissue cyst stage of the parasite. We do not know also whether the drug concentrations used in vitro correspond to therapeutic concentration reached in vivo. The only available data concern plasma levels of antipsychotics in usual clinical doses [15, 25]. For example, zuclopenthixol blood levels varies from 4 to 50 ng / mL ( 0.01 to 0.12  $\mu$ M) and fluphenazine from 1 to 10 ng / mL (0.02 to 0.2  $\mu$ M), which is well below the concentrations reported here, as these antipsychotic are classified in “low level drugs”, contrary to thioridazine, which blood levels rank from 0.5 to 5.4  $\mu$ M[15]. Thioridazine is a phenothiazine that was found by Goodwin et al to have a high antitoxoplasmic activity (IC 50 =1.2  $\mu$ M) but is not available in a soluble form in France, and is unfortunately rarely prescribed. However, another important point is that antipsychotics may accumulate in the brain and intracerebral concentrations may be much higher than plasmatic ones. Another limitation to the application of these in vitro findings is that these molecules give rise to many active derivatives when administered in humans, which can modify the drug anti-toxoplasmic activity.

## Conclusion

We have confirmed one major finding of Goodwin et al, i.e that fluphenazine has a high anti-toxoplasmic activity, but also showed that this effect is not phenothiazine class-specific. We found indeed that another first generation antipsychotic, zuclopenthixol, has a high anti-toxoplasmic activity, which may be a finding of interest for further research on *Toxoplasma* in schizophrenia. This study raises some potentially important questions that could be the focus of future research. Further studies are warranted to determine if these in vitro findings have potential implications in treatment of *Toxoplasma* infection among patients with schizophrenia and bipolar disorders. These findings may be of major interest for the choice of the first-line antipsychotic drug, in the context of emergent biomarkers for mental illnesses and the urgent need for developing personalized medicine.

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